FIELD TRIAL OF ATTENUATED SALMONELLA TYPHI LIVE ORAL VACCINE Ty21a IN LIQUID AND ENTERIC-COATED FORMULATIONS AND EPIDEMIOLOGICAL SURVEY FOR INCIDENCE OF DIARRHEA DUE TO SHIGELLA SPECIES

FINAL REPORT

MYRON M. LEVINE

APRIL 30, 1992

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-87-C-7022

Center for Vaccine Development
University of Maryland School of Medicine
10 S. Pine Street
Baltimore, Maryland 21201

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 30, 1992	Final 13 Feb 87 - 1 Apr 92

4. TITLE AND SUBTITLE Field Trial of Attenuated Salmonella typhi Live Oral Vaccine Ty21a in Liquid and Enteric-Coated Formulations and Epidemiological Survey for Incidence of Diarrhea due to Shigella Species

5. FUNDING NUMBERS
DAMD17-87-C-7022
0604807A
3S464807D849 AA
DA312324

6. AUTHOR(S)
Myron M. Levine

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Center for Vaccine Development
University of Maryland School of Medicine
10 S. Pine Street
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

By studying the epidemiology and prevention of enteric infections in cohorts of children in a less-developed area, one can gain insights that are directly applicable to the prevention of enteric infections in adult military travelers. Santiago, Chile was deemed a particularly attractive site to carry out studies of the epidemiology of enteric infections because a wide array of diarrheal pathogens were known to be prevalent. There also exists in Santiago an excellent health care infrastructure, as well as motivated and well-trained microbiologists and epidemiologists.

The enteric diseases field project in Santiago, Chile had four broad objectives: 1) to directly compare in a large-scale, randomized, placebo-controlled field trial, the efficacy of live oral typhoid vaccine, Ty21a, when administered in a liquid suspension or in enteric-coated capsules; 2) to intensively

14. SUBJECT TERMS
RA 1; Typhoid; Vaccine; Diarrhea; Shigella

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

19. (continued)

sudy the epidemiology of endemic *Shigella* infections in children residing in a low socioeconomic level community in Santiago; 3) to develop, modify, standardize, and transfer to Chile practical DNA probe methods that would allow the processing of large numbers of clinical specimens to detect the various categories of *E. coli* associated with diarrheal disease; and 4) to study the epidemiology of diarrheal disease due to the various categories of diarrheagenic *E. coli*.

Results of three years of surveillance of the direct comparison of formulations of Ty21a unequivocally demonstrate that the liquid formulation is significantly superior.Over three years of surveillance, this formulation conferred 77% protection against confirmed typhoid fever.

Shigella infections were studied in an age cross-sectional cohort of children in a low socioeconomic level community in Area Oriente, Santiago. *Shigella* accounted for 10% of all episodes of diarrhea in the prospectively-followed cohort. In the first five years of life a child had a 67% chance of experiencing shigellosis. Three serotypes, *S. sonnei*, *S. flexneri* 2A, and *S. flexneri* 6, accounted for 79% of the episodes of shigellosis. An initial episode of *Shigella* diarrhea did not diminish overall the risk of subsequent shigellosis but did diminish by an impressive 72% ($p=0.05$) the risk of subsequent illness due to the homologous serotype.

Enterotoxigenic *E. coli* (ETEC) is the single most important cause of travelers' diarrhea and is a major cause of pediatric diarrhea in less-developed countries. Other categories of *E. coli* also cause diarrhea. Testing *E. coli* isolates from clinical specimens to determine if they are representative of one of the categories of diarrheagenic *E. coli* is a daunting task if large numbers of specimens must be processed. The advent of DNA probes to detect some categories of diarrheagenic *E. coli* by using hybridization techniques to identify virulence genes offered the theoretical possibility that this approach could expand to develop a general method that could be applied to identify all categories of diarrheagenic *E. coli*. We developed a practical and economical method that uses non-radioactive DNA proves to identify diarrhea-causing *E. coli*. This methodology was transferred to and standardized in our field laboratory in Santiago, Chile.

Two cohorts of children were prospectively followed to detect episodes of diarrheal illness and to identify diarrheagenic *E. coli* in the ill children and in specimens from age-matched controls. Of particular interest are the data on ETEC infections in Santa Julia. Isolates expressing only LT were the most common toxin type encountered. However, the relative risk of isolation of LT/ST and ST-only strains was notably higher than the LT-only strains, suggesting greater pathogenicity for LT/ST and ST-only strains. It is also of interest that a known fimbrial colonization factor was found to be expressed by almost all LT/ST strains and by the great majority of ST-only strains but by few LT-only strains.

FOREWORD

For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

BODY OF THE REPORT

BACKGROUND

Enteric infections, including diarrheal diseases, dysenteries, and typhoid fever, have historically constituted major causes of morbidity among military personnel deployed under field conditions. These infectious disease threats have persisted during recent conflicts in less-developed areas of the world where United States military personnel were involved. For example, in Viet Nam, diarrheal diseases were a significant source of morbidity (1). Shigellosis was an important clinical problem faced by U.S. Marines during their deployment in Lebanon in the early 1980s. Finally, during the Desert Shield and Desert Storm campaigns in the Arabian Peninsula, diarrheal disease was the most important cause of morbidity among U.S. soldiers (2,3).

Typhoid Fever

The use of parenteral inactivated whole cell typhoid vaccines has virtually eliminated typhoid fever as a military threat. For example, during the conflict in Viet Nam, typhoid fever due to antibiotic-resistant *Salmonella typhi* was a prominent clinical problem in the unvaccinated civilian population, whereas U.S. military personnel (4) (who were vaccinated) were virtually unaffected. However, while the killed whole cell parenteral typhoid vaccine is capable of conferring significant protection, it is one of the most reactogenic of all licensed vaccines, causing febrile reactions in approximately 25% of recipients and local adverse reactions in 50% (5-7). Thus, if troops are inoculated with this vaccine shortly before being deployed, a proportion may become temporarily incapacitated because of systemic adverse reactions caused by the vaccine. In the 1970s, results of studies in volunteers (8) and of a preliminary field trial in Egypt (9) that utilized attenuated *S. typhi* strain Ty21a as a live oral vaccine gave notice that significant protection against typhoid fever could be achieved without causing adverse reactions. Despite the promising results obtained in these early trials, it was clear that considerable further field

research would have to be carried out with Ty21a to resolve critical issues that included the choice of formulation and the immunization schedule necessary to optimize the efficacy of Ty21a.

Shigella

Diarrheal infections due to *Shigella* are usually second in frequency to enterotoxigenic *E. coli* infections among travelers from industrialized areas to less-developed areas of the world, including U.S. military travelers (2,3). However, the clinical presentation of *Shigella* infections is often markedly more severe than other etiologic agents that cause travelers' diarrhea. Full-blown *Shigella* dysentery often begins with high fever, toxemia, severe abdominal cramps, and a short course of watery diarrhea that is then followed by overt dysentery (scanty stools of gross blood and mucus) accompanied by tenesmus. Individuals who suffer the more severe forms of *Shigella* dysentery are incapacitated for several days, despite the prompt initiation of appropriate antibiotics. Certain antibiotics can significantly shorten the course of clinical illness if the causative *Shigella* organisms are sensitive. Regrettably, the history of therapy of this infectious disease since the 1940s shows that, within a few months or years, *Shigella* organisms develop resistance to each new antibiotic that initially demonstrates clinical efficacy.

For the reasons cited above, the development of safe and effective vaccines to prevent shigellosis is a high priority. Progress in the development of such vaccines has been slow. In practical terms, little progress has been made since the streptomycin-dependent and T_{32} live oral *Shigella* vaccines were developed and widely employed in Eastern Europe in the 1960s (10-14). Information on infection-derived immunity can help direct the development of *Shigella* vaccines. Some information of this nature is available from re-challenge studies in volunteers but is limited to serogroup-homologous re-challenge (15,16). An ideal, albeit difficult, way to obtain data on the existence of serogroup-homologous and serogroup-heterologous immunity to *Shigella* is to carry out observational field studies of endemic shigellosis among children living in high incidence

areas in less-developed countries. Such studies would also result in identification of a site where field trials of efficacy of *Shigella* vaccines could be carried out under conditions of natural challenge.

Diarrheal Disease due to Enterotoxigenic *E. coli* and Other Categories of Diarrheagenic *E. coli*

Enterotoxigenic *Escherichia coli* is the most prominent cause of travelers' diarrhea, often accounting for up to 50% of cases (17,18). Enterotoxigenic *Escherichia coli* is also an important cause of diarrheal illness in young children living in developing countries. When one examines the toxin type and antigenic make-up of enterotoxigenic *E. coli* isolates from large studies of travelers diarrhea or of endemic diarrhea, one is impressed with the considerable heterogeneity seen (19,20). At first glance, such antigenic diversity might lead to pessimism over the likelihood of being able to achieve broad-spectrum immunity with vaccines to prevent diarrhea due to enterotoxigenic *E. coli*. On the other hand, epidemiologic studies in less-developed areas of the world show children appear to acquire broad immunity to enterotoxigenic *E. coli* as a consequence of suffering several clinical (or sub-clinical) infections. Natural immunity to enterotoxigenic *E. coli* can be based on natitoxin that neutralizes the effect of heat-labile neterotoxin (heat-stable entertoxin does not elicit neutralizing antitoxin) or anti-colonization immunity based on the appearance of antibodies to fimbrial colonization factors. Thus, a careful analysis of the toxin type and the expression of fimbrial colonization factor antigens among a large sample of enterotoxigenic *E. coli* isolates from an endemic area can provide important insights to direct vacine development. In particular, such information would identify the most important antigens that should be included in candidate vaccines and of the likelihood of achieving broad protection.

Santiago, Chile as a Site for Field Studies of Enteric Infections

Because they typically lack background immunity to enteric pathogens such as *Shigella* and enterotoxigenic *Escherichia coli*, when U.S. soldiers are deployed to less-developed areas of the world they often experience high rates of enteric disease morbidity. By studying the epidemiology and prevention of enteric infections in cohorts of children in a less-developed area, one can gain insights that are directly applicable to the prevention of enteric infections in adult military travelers.

Santiago, Chile was deemed a particularly attractive site to carry out studies of the epidemiology of enteric infections because a wide array of diarrheal pathogens were known to be prevalent, including shigellosis. Moreover, typhoid fever was highly endemic, offering the possibility of evaluating new candidate typhoid vaccines in the same geographic area. Finally, there exists in Santiago, Chile an excellent health care infrastructure, as well as motivated and well-trained microbiologists and epidemiologists. For these reasons, the studies to be described below were carried out in that geographic site, in collaboration with the Ministry of Health and the University of Chile.

BROAD OBJECTIVES OF THE PROJECT

The enteric diseases field project in Santiago, Chile had four broad objectives:

1) To directly compare, in a large-scale, randomized, placebo-controlled field trial, the efficacy of live oral typhoid vaccine, Ty21a, when administered in a liquid suspension or in enteric-coated capsules.

2) To intensively study the epidemiology of endemic *Shigella* infections in children residing in a low socioeconomic level community in Santiago. This community might thereafter serve as the site for field trials of efficacy of candidate *Shigella* vaccines or

other interventions against shigellosis (e.g., milk immunoglobulin concentrates containing *Shigella* antibodies).

3) To develop, modify, standardize and transfer to Chile practical DNA probe methods that would allow the processing of large numbers of clinical specimens to detect the various categories of *E. coli* associated with diarrheal disease.

4) To study the epidemiology of diarrheal disease due to the various categories of diarrheagenic *E. coli*.

In the ensuing paragraphs we will summarize the results of the studies that pursued these four broad objectives.

COMPARISON OF THE EFFICACY OF LIQUID SUSPENSION VERSUS ENTERIC-COATED CAPSULE FORMULATION OF Ty21a, LIVE ORAL TYPHOID VACCINE

Ty21a is an attenuated strain of *S. typhi* that was prepared in the 1970s by chemical mutagenesis of a wild type strain (21). As of the mid 1980s, Ty21a had varied success when used as a live oral vaccine to prevent typhoid fever. In the first field trial in Alexandria, Egypt, three doses of vaccine (every other day interval) administered as a liquid suspension after administration of buffer gave 96% protection over three years of follow-up (9). In a much larger field trial in Area Occidente, Santiago, Chile, three doses of vaccine in enteric-coated capsule formulation conferred 67% protection during three years of follow-up (22) and 63% protection over seven years of follow-up (23). Several possible explanations could account for the apparent difference in level of efficacy observed in the Alexandria versus the Area Occidente field trials. These include differences in the immune response to Ty21a based on the distinct genetic backgrounds of the populations studied; differences in the antigenic structure of the endemic S.

typhi strains in Egypt versus Chile; differences in modes of transmission (which might affect inoculum size); statistical considerations; lastly, a liquid formulation of vaccine such as used in Egypt might be superior to enteric-coated capsules. Since formulation was the one variable that could be directly compared in a randomized, placebo-controlled, double-blind field trial, such a trial was carried out in Area Sur Oriente and Area Norte, Santiago.

Results of three years of surveillance of the direct comparison of formulations of Ty21a unequivocally demonstrate that the liquid formulation is significantly superior. Over three years of surveillance this formulation of Ty21a conferred 77% protection against confirmed typhoid fever. The description of this field trial and the results of the first three years of surveillance were published in a lead article in The Lancet; a copy of this publication is attached as APPENDIX A.

We maintained surveillance for two additional years in this field trial to give a total of five years of surveillance. The results of this additional follow-up are summarized in Table 1. There was no fall-off in protective efficacy during the fourth and fifth years of surveillance.

It should be noted that, largely based on results of the four U.S. Army Medical Research and Development Command-supported field trials of efficacy of Ty21a that were carried out in Santiago, Chile (22,24-26), this oral typhoid vaccine was licensed by the U.S. Food and Drug Administration for prevention of typhoid fever in travelers. The Armed Forces Epidemiological Board has recommended that Ty21a be used for immunizing U.S. troops who would be deployed to less-developed areas of the world where sanitation is compromised and risk of transmission *S. typhi* poses a real threat.

THE EPIDEMIOLOGY OF ENDEMIC SHIGELLA INFECTIONS

Shigellosis constitutes one of the most disruptive infectious disease threats faced by U.S. troops who are deployed in less-developed areas of the world under field conditions. During

Desert Shield and Desert Storm, *Shigella* was second to enterotoxigenic *E. coli* as a recognized etiologic agent of diarrheal infections in U.S. troops. Shigellosis, particularly when it occurs as full blown bacillary dysentery, is a clinically severe enteric infection that is especially disabling.

The development of successful immunizing agents against shigellosis is a high priority. There exist four species (also referred to as groups) of *Shigella, S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei.* Multiple serotypes and subtypes exist in all but the *S. sonnei* group. Little has been published about infection-derived immunity in shigellosis or about its serogroup-specificity if it exists.

Shigella infections were studied in an age cross-sectional cohort of children in a low socioeconomic level community in Area Oriente, Santiago. The community of Santa Julia is notable because the generally poor and disadvantaged population there which lives in ramshackle housing under very crowded conditions, nevertheless, has access to bacteriologically-monitored water.

A cohort of approximately 340 children less than 60 months of age was followed for 30 months with twice weekly household visits to detect diarrheal illness. When an episode of diarrhea was detected, coprocultures were obtained from the child on two consecutive days as well as from a matched control child without diarrhea. Children from Santa Julia who visited the neighborhood health center (consultorio) with diarrheal illness or who were admitted with diarrhea to the Calvo MacKenna Children's Hospital that serves Area Oriente, were also cultured for *Shigella.*

The observations on the epidemiology of *Shigella* infections in Santa Julia are contained in a publication (APPENDIX B) that appeared in the American Journal of Epidemiology. *Shigella* accounted for 10% of all episodes of diarrhea in the prospectively-followed cohort. In the first five

years of life a child had a 67% chance of experiencing shigellosis. Three serotypes, *S. sonnei*, *S. flexneri* 2a and *S. flexneri* 6, accounted for 79% of the episodes of shigellosis.

For the first time in an epidemiologic study, quantitative data were available to investigate infection-derived immunity. An initial episode of *Shigella* diarrhea did not diminish overall the risk of subsequent shigellosis but did diminish by an impressive 72% ($p = 0.05$) the risk of subsequent illness due to the homologous serotype. These field data on infection-derived immunity complement results of studies carried out in the 1960s and early 1970s in Yugoslavia with live oral *Shigella* vaccines where streptomycin-dependent *Shigella* strains were found to confer serotype-specific protection (10-12).

A PRACTICAL DNA PROBE METHOD TO IDENTIFY THE DIFFERENT CATEGORIES OR DIARRHEAGENIC E. COLI

Enterotoxigenic *E. coli* (ETEC) is the single most important cause of travelers' diarrhea and is a major cause of pediatric diarrhea in less-developed countries (20). Other categories of E. coli also cause diarrhea, including enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC) and diffuse adherence *E. coli* (DAEC) (20,27,28). Testing *E. coli* isolates from clinical specimens to determine if they are representative of one of the categories of diarrheagenic *E. coli* is a daunting task if large numbers of specimens must be processed. Were this to be attempted by detecting phenotypic expression of virulence traits, the number of different tests for distinct enterotoxins, invasion, adherence factors, etc., would make such an undertaking logistically cumbersome, impractical, time-consuming, labor-intensive and terribly expensive. The advent of DNA probes to detect some categories of diarrheagenic *E. coli* by using hybridization techniques to identify virulence genes offered the theoretical possibility that this approach could expanded to develop

a general method that could be applied to identify all categories of diarrheagenic *E. coli*. Lacking at the time the project started were sensitive and specific DNA probes for all the distinct categories of diarrheagenic *E. coli* (some were available) and a non-radioactive signal technique that could be transferred to a laboratory in a less-developed country for routine processing of large numbers of clinical specimens.

APPENDIX C, a publication in the Journal of Clinical Microbiology, decribes a practical and economical method that uses non-radioactive DNA probes to identify diarrhea-causing *E. coli*. This methodology was transferred to and standardized in our field laboratory in Santiago, Chile.

INTENSIVE STUDIES OF THE EPIDEMIOLOGY OF ENTERIC ILLNESS DUE TO THE DIFFERENT CATEGORIES OF DIARRHEAGENIC E. COLI

Two cohorts of children were prospectively followed to detect episodes of diarrheal illness and to identify diarrheagenic *E. coli* in the ill children and in specimens from age-matched controls. In addition to the age cross-sectional cohort that was followed for 30 months, a newborn cohort was followed prospectively until all the infants reached at least 24 months of age. Moreover, children less than 60 months of age who visited the local Santa Julia health center or who were admitted to the Calvo MacKenna Children's Hospital with diarrheal illness were also cultured to detect diarrheagenic *E. coli*.

A draft manuscript summarizing the extensive studies on diarrheagenic *E. coli* is attached as APPENDIX D. Of particular interest are the data on ETEC infections in Santa Julia. Isolates expressing only LT were the most common toxin type encountered. However, the relative risk of isolation of LT/ST and St-only strains was notably higher than the LT-only strains, suggesting greater pathogenicity for LT/ST and ST-only strains. It is also of interest that a known fimbrial colonization factor was found to be expressed by almost all LT/ST strains and by the great

majority of ST-only strains but by few LT-only strains. Taken together, these results bode well for the expectation that a future ETEC vaccine based on stimulating and anti-colonization factor and anti-LT immunity will have the potential to provide broad-spectrum protection.

LITERATURE CITED

1. Griffin RB Jr, Gaines S. Diarrhea in a U.S. battle group in Thailand. Milit Med 1964; 129:546-550.

2. Hyams KC, Bourgeois AL, Merrell BR, Rozmajl P, Escamilla, Thornton SA, Wasserman GM, Burke A, Echeverria P, Green KY, Kapikian AZ, Woody JN. Diarrheal disease during Operation Desert Shield. N Eng J Med 1991;325:1423-1428.

3. Gasser RA Jr, Magill AJ, Oster CN, Tramont EC. The threat of infectious disease in Americans returning from Operation Desert Storm. N Eng J Med 1991; 224:859-864.

4. Butler T, Linh NN, Arnold, Pollack M. Chloramphenicol-resistant typhoid fever in Vietnam associated with R factor. Lancet 1973; II:983-986.

5. Yugoslav Typhoid Commission. A controlled field trial of the effectiveness of acetone-dried and inactivated and heat-phenol- inactivated typhoid vaccines in Yugoslavia. Bull WHO 1964; 30:623-230.

6. Hejfec LB, Salmin LV, Lejtman MZ, et al. A controlled field trial and laboratory study of five typhoid vaccines in the USSR. Bull WHO 1966; 34:321-339.

7. Ashcroft MT, Morrison-Ritchie J, Nicholson CC. Controlled field trial in British Guyana school-children of heat-killed-phenolized and acetone-killed lyophilized typhoid vaccines. Amer J Hyg 1964; 79:196-206.

8. Gilman RH, Hornick RB, Woodward WE, et al. Immunity in typhoid fever: evaluation of Ty21a - an epimeraseless mutant of S. typhi as a live oral vaccine. J Infect Dis 1977; 136:717-723.

9. Wahdan MH, Serie C, Cerisier Y, Sallam S, Germanier R. A controlled field trial of Live Salmonella typhi strain Ty21a oral vaccine against typhoid: three year results. J Infect Dis 1982; 145:292-295.

10. Mel DM, Terzin AL, Vuksic L. Studies on vaccination against bacillary dysentery. 3. Effective oral immunization against Shigella flexneri 2a in a field trial. Bull WHO 1965; 32:647-655.

11. Mel DM, Arsic BL, Nikolic BD, Radovanovic ML. Studies on vaccination against bacillary dysentery. 4. Oral immunization with live monotypic and cmbined vaccines. Bull WHO 1968; 39:375-380.

12. Mel DM, Arsic BL, Radovanovic ML, Litvinjenko S. Live oral Shigella vaccine: vaccination schedule and the effect of booster dose. Acta Microbiol Acad Scient Hung 1974; 21:109-114.

13. Istrati G, Meitert T, Ciufecu C. Recherches sur l'immunite active de l'homme dans la dysenterie bacillaire. Arch Roumaines Pathol Exp Microbiol 1965;24:677-686.

14. Meitert T, Istrati G, Sulea T, et al. Prophuylaxie de la dysenterie bacillaire par le vaccine vivant antidysenterique dans une collectivite d'enfants neuropsychiques. Arch Roum Exp Microbiol 1973; 32:35-44.

15. DuPont HL, Hornick RB, Snyder MJ, Libonati JP, Formal SB, Gangarosa EJ. Immunity in shigellosis: Protection induced by oral ive vaccine or primary infection. J Infect Dis 1972; 125:12-16.

16. Herrington D, Van de Verg L, Formal SB, Hale TL, Tall BD, Cryz SJ, Tramont EC, Levine MM. Studies in volunteers to evaluate candidate Shigella vaccines: further experience with a bivalent Salmonella typhi-Shigella sonnei vaccine and protection conferred by previous Shigella sonnei disease. Vaccine 1990; 8:353-357.

17. Merson MH, Morris GK, Sack DA, et al. Travelers' diarrhea in Mexico. A prospective study of physicians and family members attending a congress. N Eng J Med 1976; 294:1299-1305.

18. Black RE. Pathogens that cause travelers' diarrhea in Latin America and Africa. Rev Infect Dis. 1986; 8 supplement:S131-111.

19. Levine MM, Ristaino P, Sack RB, Kaper JB, Orskov F, Orskov I. Colonization factor antigens I and II and type 1 somatic pili in enterotoxigenic Escherichia coli: relation to enterotoxin type. Infect Immun 1983; 39:889-897.

20. Levine MM. Escherichia coli that cause diarrhea: Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J Infect Dis 1987;155:377-389.

21. Germanier R, Furer E. Isolation and characterization of gal E mutant Ty21a of Salmonella typhi: a candidate strain for a live oral typhoid vaccine. J Infect Dis 1975; 141:553-558.

22. Levine MM, Ferreccio C, Black RE, Chilean Typhoid Committee, Germanier R. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet 1987; I:1049-1052.

23. Levine MM, Ferreccio C, Ortiz E, Cryz S. Unpublished data.

24. Levine MM, Ferreccio C, Cryz S, Ortiz E. Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in a randomized controlled field trial. Lancet 1990; 336:891-894.

25. Black RE, Levine MM, Ferreccio C, Clements ML, Lanata C, Rooney J, Germanier R, and the Chilean Typhoid Committee. Efficacy of one or two doses of Ty21a Salmonella typhi vaccine in enteric-coated capsules in a controlled field trial. Vaccine 1990; 8:81-84.

26. Ferreccio C, Levine MA, Rodriguez H, et al. Comparative efficacy of two, three, or four doses of Ty21a live oral typhiod vaccine in enteric coated capsules. J Infect Dis 1989;159:766-769.

27. Bhan MK, Raj P, Levine MM, Kaper JB, Bhandari N, Srivastava R, Kumar R, Sazawal S. Enteroaggregative Escherichia coli associated with persistent diarrhea in a cohort of rural children in India. J Infect Dis 1989; 159:1061-1064.

28. Giron J, Jones T, Millan-Velasco F, Muñoz-castro E, Fry J, Frankel G, Moseley S, Baudry B, Kaper JB, Schoolnik GK, Riley LW. Diffuse-adhering Escherichia coli (DAEC) as a putatuve cause of diarrhea in Mayan children in mexico. J Infect Dis 1991; 164:693-703.

Table 1. Comparison of the efficacy of three doses of Ty21a live oral typhoid vaccine administered to Chilean schoolchildren in enteric-coated capsules or as a liquid suspension. Results of five years of surveillance

	Enteric Capsules (34,696)	Liquid Suspension (36,623)	Placebo (10,302)
YEAR 1 (11/86 - 10/87)			
Cases	20	7	9
Incidence/10^5	57.6	19.1	87.4
Efficacy	34%	78%	-
YEAR 2 (11/87 - 10/88)			
Cases	19	6	11
Incidence/10^5	54.8	16.4	106.8
Efficacy	49%	85%	-
YEAR 3 (11/88 - 10/89)			
Cases	24	10	8
Incidence/10^5	69.2	27.3	77.7
Efficacy	11%	69%	-
YEAR 4 (11/89 - 10/90)			
Cases	20	5	8
Incidence/10^5	57.6	13.7	77.7
Efficacy	26%	82%	-
YEAR 5 (11/90 - 10/91)			
Cases	18	6	7
Incidence/10^5	51.9	16.4	67.9
Efficacy	24%	76%	-
YEARS 1 - 5 (11/86 - 10/91)			
Cases	101	34	43
Incidence/10^5	291.1	92.8	417.4
Efficacy	30%	78%	-
95% confidence interval			

APPENDIX A

MEDICAL SCIENCE

Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in randomised controlled field trial

MYRON M. LEVINE CATTERINE FERRECCIO STANLEY CRYZ
EDITH ORTIZ

In a randomised, double-blind, controlled field trial in Santiago, Chile, 81 621 schoolchildren aged 5–19 years received three doses, within a week, of attenuated *Salmonella typhi* oral vaccine Ty21a in enteric-coated capsules or in a new liquid suspension, or placebo. Over 36 months of surveillance, the liquid formulation (76·9% vaccine efficacy) was significantly superior to the enteric-coated capsules (33·2% vaccine efficacy). The liquid formulation protected young children (5–9 years) (efficacy 82·3%) as well as older children (efficacy 69·3%), whereas the capsules significantly protected only older children. The liquid suspension was easy to prepare by mixing lyophilised vaccine with buffer in water and was easily adminstered, even to the youngest children. Thus, the liquid formulation of Ty21a is preferable to enteric-coated capsules.

Lancet 1990; 336: 891–94.

Introduction

About 33 million cases of typhoid fever occur annually world wide.[1] School-age children in less-developed countries,[2-5] travellers to such regions from industrialised countries,[6] and clinical microbiology technicians[7] are at particular risk. Ty21a, an attenuated strain of *Salmonella typhi* that lacks the Vi polysaccharide,[8] used as a live oral vaccine, is an advance in immunoprophylaxis against typhoid fever because it protects significantly without causing adverse reactions.[9-14] However, in field trials of Ty21a the formulation of vaccine used and the number of doses given greatly affected the level of efficacy achieved.[10-15]

In the first controlled field trial of Ty21a, in Alexandria, Egypt, three doses (10^9 viable vaccine organisms per dose) given on alternate days gave 96% protection against culture-confirmed typhoid fever during 3 years of surveillance in 6–7-year-old children.[10,15] They were given 1·0 g sodium bicarbonate to neutralise gastric acid followed 1–3 min later by a liquid suspension of Ty21a or placebo (prepared by reconstituting lyophilised vaccine or placebo with a diluent just before administration).[10,15] Unfortunately, the formulation used was impractical for mass production.

We tested a formulation that does not require buffer, because it consists of enteric-coated capsules that open only in a surrounding pH of 6·0 or more, in schoolchildren in a large-scale field trial in Area Occidente, Santiago, Chile.[11] Three doses of Ty21a in enteric-coated capsules given within a week provided 67% protection for the first 3 years of follow-up and 66% protection for 5 years.[11,13] In another field trial in two other administrative areas of Santiago an immunisation schedule of four doses of Ty21a given within 8 days was significantly more protective than 3 doses.[12] On the basis of these data, the Food and Drug Administration in the USA licensed the enteric-coated capsule formulation of Ty21a with a recommended immunisation schedule of four doses to be given on days 1, 3, 5, and 7.

Although the enteric-coated capsule formulation of Ty21a is practical and moderately effective, epidemiologists and public health officials remain intrigued by the higher 3-year efficacy (96%) with the liquid formulation.[10] Possible explanations of the difference include:[14] differences in the immune response to Ty21a caused by genetic variations in the two study populations; differences in the antigenic

ADDRESSES: Center for Vaccine Development and Division of Geographic Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, USA (Prof M. M. Levine, MD, C. Ferreccio, MD); Typhoid Fever and Enteric Infections Control Program, Ministry of Health, Santiago, Chile (C. Ferreccio, E. Ortiz, RN); and Swiss Serum and Vaccine Institute, Berne, Switzerland (S. Cryz, PhD). Correspondence to Prof M. M. Levine, Center for Vaccine Development, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201, USA.

make-up of endemic *S typhi* strains; differences in the modes of transmission of *S typhi* resulting in ingestion of higher inocula in Santiago (mainly food-borne) than in Alexandria (mainly water-borne); statistical considerations; and inherent superiority of a liquid suspension over an enteric-coated capsule formulation.

Formulation is the one variable amenable to evaluation in a prospective field trial and the development of a new liquid formulation made such a comparative trial feasible. We have carried out a randomised, double-blind, controlled field trial in two administrative areas of Santiago, Chile, to compare directly the efficacy of Ty21a in enteric-coated capsules and in a liquid suspension.

Subjects and methods

Advantages of carrying out field trials of typhoid vaccines in Santiago, Chile, include a high incidence of typhoid fever in schoolchildren,[4,11,12] an excellent health care infrastructure,[11,12,16] and prior experience in organising large-scale typhoid vaccine field trials.[11,12,17] The trial design and consent procedures were approved by ethical review committees of the University of Maryland School of Medicine, the World Health Organisation, Geneva, and the US Department of Defense, Washington, DC.

The Ministries of Health and of Education in Chile ensured that parents were informed of the trial by distributing an information brochure. Permission to enrol each child was then requested by means of consent forms and the parental response was recorded.

The need to measure the absolute efficacy of each formulation, as well as their relative efficacy, made it necessary to include a control group; the size of this group was kept to the minimum number required for statistical significance. On the basis of estimates of vaccine efficacy for each formulation and predictions of incidence rates in the control and vaccine groups, we calculated that we needed 1 placebo child for every 7 vaccine recipients. When this trial started, Ty21a was not a routine immunisation for schoolchildren in Santiago or elsewhere in Chile. Thus, children in the control group were not deprived of a routine vaccine that they would otherwise receive. The placebo preparation given to the controls contained viable *Lactobacillus acidophilus*.

Hydroxypropylmethylcellulosephthalate was the enteric coating used to make the capsules acid resistant. Such capsules resist gastric acid (pH 1·5) for at least 2 h but dissolve within 10 min in artificial intestinal fluid of pH 6·0 or more.[11]

The liquid formulation of vaccine (or placebo) consisted of two aluminium foil packets; one contained lyophilised viable organisms ($1–3 \times 10^9$) and 25 mg aspartame and the other a powdered buffer (2·65 g sodium bicarbonate and 1·65 g ascorbic acid). To prepare a dose, the contents of the two packets were mixed in a cup with 100 ml water.

During the 2 years before the trial, the annual incidence of typhoid fever in children aged 5–19 years in Area Sur Oriente was 103 cases per 10^4. The maximum population of schoolchildren available for this field trial was 174 589 (137 767 5–19-year-olds in Area Sur Oriente and 36 822 5–9-year-olds in Area Norte). Based on experience in our three previous field trials in Santiago, a parental consent rate of 60–96% could be expected. Thus, as few as 104 153 or as many as 166 822 schoolchildren might be available for randomisation.

Assumptions in the calculation of sample sizes included 3 years of surveillance, a statistical power of 80%, $p < 0·025$ for detection of absolute efficacy compared with the control group (single-tail hypothesis), $p < 0·05$ (two-tailed) for detection of relative efficacy between the two formulations of vaccine, with enteric-coated capsule Ty21a estimated to provide 60% efficacy for at least 3 years. Based on these assumptions, with an estimated incidence of 90 cases of typhoid per 10^4 in the *Lactobacillus* control group, inclusion of 10 743 children in the control group and 24 174 children in each of the two vaccine groups would allow detection of an absolute vaccine efficacy of at least 60% and of a significant difference between the formulations if the liquid formulation were 25% more effective than the capsules.

TABLE I—COMPARISON OF EFFICACY

—	Liquid	Enteric capsules	Placebo
No of children	36 623	34 696	10 302
Cases	23	63	28
Incidence/10^4	63	182	272
Efficacy (95%CI)*	76·9 (60–87)%	33·2 (0–57)%	..
No of classes	2369	2367	687
Classes with typhoid	23	61	27
Classes with typhoid per 100 classes	0·97	2·57	3·93
Efficacy (95% CI)*	75·3 (56–85)%	34·6 (0–59)%	..

*Vaccine efficacy = $\frac{\text{incidence in placebo group} - \text{incidence in vaccine group}}{\text{incidence in placebo group}} \times 100\%$

In Santiago, peak transmission of typhoid fever and the vast majority of cases occur during the summer season from mid-December to mid-March when schools are not in session.[4,11,12] Vaccination was therefore carried out in September and October, several months before onset of the typhoid season. To simplify the logistics of the school-based vaccination, randomisation was done by classroom—all children of consenting parents within a class received the same vaccine or placebo regimen.

The packets and the enteric-coated capsules containing placebo appeared identical to those containing vaccine. Trial organisers, those carrying out vaccinations, the children and their parents, and health care providers were unaware which preparation (ie, vaccine or placebo) had been given to any child. Since the number of control children was much smaller (an eighth) than the number who were to receive vaccine, to maintain double blindness each formulation was evenly divided into eight separate letter codes; one letter represented the control preparation and the other seven vaccine. The code was kept by the Diarrhoeal Diseases Control Programme of WHO until 36 months of surveillance had been completed. Results were analysed by the chi-square test for statistical significance.

The vaccination was carried out by trained health workers in the classrooms during September and October, 1986; computerised data files were generated from the completed class lists. Surveillance began in November, 1986. Approximately 90% of health care visits in Area Sur Oriente and Area Norte are in facilities of the National Health Service where intensive surveillance could be maintained; the remaining visits are to private practitioners. Physicians and nurses were kept aware of the importance of obtaining cultures from suspected cases of typhoid fever by means of clinical conferences, letters from the Ministry, and weekly visits by surveillance nurses from the Typhoid Fever Control Program. Only confirmed cases—those from which *S typhi* was isolated from blood, bone marrow, or bile-stained duodenal fluid cultures[11,12,18]—were used to calculate vaccine efficacy. Considerable resources were therefore expended to ensure bacteriological confirmation of suspected cases. From children in hospital three 4 ml blood cultures were obtained, sometimes accompanied by a bone-marrow culture.[18] Two 6 ml blood cultures were taken 30 min apart from outpatients presenting to health centres with suspected typhoid fever.[11] Suspect colonies were examined by standard biochemical and serological techniques.[18]

Results

Parents of 64% of the eligible schoolchildren gave consent for their children to participate; 95 910 of these children received at least one dose of vaccine or the *Lactobacillus* placebo (64 413 in Area Sur Oriente and 31 497 in Area Norte). The children in Area Sur Oriente ranged in age from 5 to 19 years. In contrast, since a previous field trial had been carried out in Area Norte in 1982,[14,17] participation there was limited to children who started school after the 1982 vaccine trial (5–9 year olds). 81 621 children received the full three scheduled doses of vaccine or placebo. During the vaccination period there was no increase in absenteeism

TABLE II—ANALYSIS OF EFFICACY BY AGE

	This study			Alexandria, Egypt[10]		Area Occidente, Santiago[11]	
—	Liquid	Enteric capsules	Placebo	Liquid	Placebo	Enteric capsules	Placebo
Age 5–9 yr							
No of children	22 586	21 128	5989	16 486	15 902	7034	7193
Cases	10	44	15	1	22	10	25
Incidence/10^5	44·3*	208	251	6·1†	138	142‡	348
Efficacy (95% CI)	82·3 (61–92)%	16·9 (0–53)%	..	95·6 (77–99)%	..	59·1 (16–80)%	..
Age ≥ 10 yr							
No of children	14 037	13 568	4313	..	..	15 134	14 711
Cases	13	19	13	..	..	13	45
Incidence/10^5	92·6‡	140‡	301	..	..	85·9†	306
Efficacy (95% CI)	69·3 (35–86)%	53·5 (7–77)%	..	..	..	71·9 (48–55)%	..

Data from all three field trials represent results from 36 months of surveillance of schoolchildren who received three doses of vaccine or placebo within 1 week. In the Alexandria trial only 6 and 7-year-olds were vaccinated
For comparisons with appropriate placebo groups. *$p < 0.00001$. †$p < 0.0001$. ‡$p < 0.025$.

or any significant rise in febrile or gastrointestinal illnesses, and no cases of typhoid fever were recorded among the participating children.

The results of 36 months of surveillance are summarised in table I. The two placebo groups were combined for the calculation of vaccine efficacy since the incidence of typhoid fever was similar in the individual groups (liquid placebo 14/5450 [257/10^5]; enteric placebo 14/4852 [289/10^5]). Both formulations of Ty21a provided significant protection against confirmed typhoid fever compared with the placebo ($p < 10^{-7}$ for liquid formulation; $p = 0.048$ for enteric capsules), but the overall level of protection was significantly higher with the liquid than with the enteric-coated capsule formulation ($p = 0.0000077$; two-tailed chi-square test).

Since we carried out randomisation by class it was important to verify that no clustering of cases occurred. Cases of typhoid fever were observed in 374 of the 5423 classes in Sur Oriente and Norte, including cases in non-participants. Only 18 of these 374 classes (4·8%) had more than 1 case of typhoid fever (17 classes had 2, and 1 class had 3); these cases occurred more than 60 days apart in all but three instances, which occurred during the school summer holiday. The distribution of cases per class did not differ from the expectations of a Poisson distribution ($p = 0.165$). To verify further the appropriateness of the randomisation, the incidence of typhoid fever was calculated as classes with typhoid cases per 100 classes vaccinated with each formulation of vaccine or with the control preparation. Again, both vaccines provided significant protection compared with the placebo ($p < 10^{-7}$ for liquid formulation, $p = 0.041$ for enteric capsules), but the liquid was significantly more effective ($p = 0.00045$).

The level of protection afforded by vaccine in enteric-coated capsules fell in the 3rd year of surveillance to 11% from 34% and 49% vaccine efficacy recorded in years 1 and 2 of follow-up. In contrast the efficacy of the liquid formulation remained above 60% (78% year 1, 85% year 2, 65% year 3).

The efficacy of each formulation of Ty21a in relation to age is shown in table II. The degree of protection provided by the liquid formulation was similar in both the young (5–9-year-old) and older (10 years and older) children. In contrast, the enteric-coated capsules conferred 53·5% protection in older children ($p = 0.06$) but there was no significant protection in the young children. The failure of enteric-coated vaccine to protect young children significantly occurred in both Sur Oriente (vaccine efficacy 16·5%) and Norte (vaccine efficacy 18·4%). In older children (table II), the vaccine efficacy afforded by the liquid formulation (69·3%) was better than that by enteric-coated capsules (51·1%) but the difference was not significant ($p = 0.2$).

Discussion

The safety record of Ty21a live oral typhoid vaccine in more than 600 000 recipients, mostly children, of more than 1·4 million doses in prospective clinical trials[13] is a substantial improvement over the highly reactogenic, parenteral killed whole cell typhoid vaccines.[14,19–22] Our trial shows that Ty21a in a liquid suspension is superior to enteric-coated capsules of the vaccine, particularly in young children. There are several possible explanations for this finding. Vaccine organisms may be hardier when reconstituted in vitro before ingestion than if they must recover from the lyophilised state immediately after release from a degraded capsule in the intestine, where they are exposed to bile acids, digestive enzymes, and partially digested food. Moreover, a liquid suspension affords the vaccine organisms contact with the tonsils, an immune system organ; this potentially important contact does not occur with vaccine in enteric-coated capsules.

In table II the data from this trial are compared with those from the two other randomised, placebo-controlled field trials of Ty21a in which similar formulations were given in the same immunisation schedule. In young children the efficacy of the liquid formulation used in Sur Oriente/Norte (82·3%) approached that (96%) seen with the liquid formulation used in Alexandria (table II); furthermore, the 95% confidence intervals surrounding these two levels of vaccine efficacy were similar.

The level of protection given by enteric-coated Ty21a in the older children in Sur Oriente/Norte was lower than that recorded in the same age group in the earlier Occidente trial (table II). However, the overlap in the confidence intervals suggests that the different results are only variations of the sort expected when independent field trials are carried out in distinct geographic sites or in the same site in different years.

The most important difference between this trial and the Area Occidente trial[11] is the poor efficacy of vaccine in enteric-coated capsules in 5–9-year-olds (16·9% *vs* 59·1% vaccine efficacy; table II), but even for this category the 95% confidence intervals overlap. Thus, the observed difference may also be due simply to chance.

In a comparison of the liquid and enteric-coated formulations of Ty21a in Plaju, Indonesia, preliminary results showed the liquid formulation to be slightly but not significantly superior.[23] The annual typhoid fever attack rate in Plaju is several-fold higher than in our trial and a different

immunisation schedule was used (three doses were given 1 week apart).

Our findings lead us to recommend the liquid formulation of Ty21a for use in endemic areas, especially for children. This formulation had better efficacy at all ages, and it was much more practical for immunising the youngest children, some of whom had difficulty swallowing the enteric-coated capsules.

Although care must be taken in extrapolating from results of a field trial in an endemic area to expectations of protection for travellers, it can be argued that the youngest children in this field trial most closely resemble travellers from industrialised countries because they have the least background immunity from prior exposure to *S typhi*. These young children were impressively protected by the liquid formulation of Ty21a. It is also known from experimental challenge studies in North American volunteers (albeit with higher doses) that Ty21a can protect against a high inoculum of pathogenic *S typhi*.[9] Together, these observations suggest that the liquid formulation of Ty21a should be preferred for immunising travellers. Ty21a is licensed in many countries but is now available only in enteric-coated capsules. A liquid formulation should be available by 1991.

We thank the staffs of the Ministries of Health and Education; Dr Ingeborg Prenzel and Dr Nancy Enriquez (Hospital Roberto del Rio) and Dr Hernan Rojas (Hospital Sotero del Rio) for bacteriological support; Dr Maria Ines Gomez and Agustin Cruz Melo for field epidemiological assistance; the staff of the Instituto de Salud Publica and the Almacen Central (Central Vaccine Storage Facility); and Viviana Sotomayor, Gloria Bernos, Leonor Astroza, and Dr Rodrigo Contreras, of the Typhoid Fever Control Program. This study was supported by a grant from the Diarrhoeal Diseases Control Programme of the World Health Organisation and by research contract DAMD17-87-C-7022 from the US Army Medical Research and Development Command.

REFERENCES

1. Institute of Medicine. New vaccine development: establishing priorities, vol II. Diseases of importance in developing countries. Washington DC. National Academy Press, 1986. Appendix D-14, Prospects for immunizing against *Salmonella typhi*: 1–10.
2. Edelman R, Levine MM. Summary of an international workshop on typhoid fever. *Rev Infect Dis* 1986; 8: 329–49.
3. Ashcroft MT. The morbidity and mortality of enteric fever in British Guyana. *W Ind Med J* 1962; 11: 62–71.
4. Levine MM, Black RE, Ferreccio C, et al. Interventions to control endemic typhoid fever: field studies in Santiago, Chile. In: Control and eradication of infectious diseases, an international symposium. Pan American Health Organisation Copublication Series no 1. Washington, DC: PAHO, 1985: 37–53.
5. Levine MM, Grados O, Gilman RH, Woodward WE, Solis-Plaza R, Waldman W. Diagnostic value of the Widal test in areas endemic for typhoid fever. *Am J Trop Med Hyg* 1978; 27: 795–800.
6. Taylor DN, Pollard RA, Blake PA. Typhoid in the United States and the risk to the international traveller. *J Infect Dis* 1983; 148: 599–602.
7. Blaser M, Hickman FW, Farmer JJ, Brenner DJ, Balows A, Feldman RA. *Salmonella typhi*: the laboratory as a reservoir of infection. *J Infect Dis* 1980; 142: 934–38.
8. Germanier R, Furer E. Isolation and characterization of gal E mutant Ty21a of *Salmonella typhi*: a candidate strain for a live oral typhoid vaccine. *J Infect Dis* 1975; 141: 553–58.
9. Gilman RH, Hornick RB, Woodward WE, et al. Immunity in typhoid fever: evaluation of Ty21a—an epimeraseless mutant of *S typhi* as a live oral vaccine. *J Infect Dis* 1977, 136: 717–23.
10. Wahdan MH, Serie C, Cerisier Y, Sallam S, Germanier R. A controlled field trial of live *Salmonella typhi* strain Ty21a oral vaccine against typhoid: three year results. *J Infect Dis* 1982; 145: 292–96.
11. Levine MM, Ferreccio C, Black RE, Germanier R, Chilean Typhoid Committee. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. *Lancet* 1987; i: 1049–52.
12. Ferreccio C, Levine MM, Rodriquez H, Contreras R, Chilean Typhoid Committee. Comparative efficacy of two, three or four doses of Ty21a live oral typhoid vaccine in enteric-coated capsules. A field trial in an endemic area. *J Infect Dis* 1989; 159: 766–69.
13. Levine MM, Taylor DN, Ferreccio C. Typhoid vaccines come of age. *Pediatr Infect Dis J* 1989; 8: 374–81.
14. Levine MM, Ferreccio C, Black RE, Tacket CO, Germanier R, Chilean Typhoid Commission. Progress in vaccines against typhoid fever. *Rev Infect Dis* 1989; 11 (suppl 3): S552–67.
15. Wahdan Serie C, Cerisier Y, Sallam S, Germanier R. A controlled field trial of live oral typhoid vaccine Ty21a. *Bull WHO* 1980; 58: 469–74.
16. Borgono JM, Corey G. 25 years of an integrated vaccination program in Chile. *Develop Biol Stand* 1978; 41: 301–06.
17. Black RE, Levine MM, Ferreccio, et al. Efficacy of one or two doses of Ty21a *Salmonella typhi* vaccine in enteric-coated capsules in a controlled field trial. *Vaccine* 1990; 8: 81–84.
18. Avendano A, Herrera P, Horwitz I, et al. Duodenal string cultures: practicality and sensitivity for diagnosing enteric fever in children. *J Infect Dis* 1986; 53: 359–62.
19. Levine M. Typhoid fever vaccines. In: Plotkin SA, Mortimer EA Jr, eds. Vaccines. Philadelphia: WB Saunders, 1989: 333–61.
20. Ashcroft MT, Morrison-Ritchie J, Nicholson CC. Controlled field trial in British Guyana school-children of heat-killed-phenolized and acetone-killed lyophilized typhoid vaccines. *Am J Hyg* 1964; 79: 196–206.
21. Heifec LB, Salmin LV, Lerman MZ, et al. A controlled field trial and laboratory study of five typhoid vaccines in the USSR. *Bull WHO* 1966; 34: 321–39.
22. Yugoslav Typhoid Commission. A controlled field trial of the effectiveness of acetone-dried and inactivated and heat-phenol-inactivated typhoid vaccines in Yugoslavia. *Bull WHO* 1964; 30: 623–30.
23. Simanjuntak C, Paleologo FP, Punjabi NH, Darmowigwoto, Soeprawato, Hoffman SL. Randomized double blind placebo controlled trial of the efficacy of Ty21a oral typhoid vaccine in Plaju, Indonesia. Abstr 1120, 28th Interscience Conference on Antimicrobial Agents and Chemotherapy, Los Angeles, California, October, 1988.

From The Lancet

Chloroform amongst thieves

The thieves have, it seems, "interrogated nature" with somewhat greater success than has attended the efforts of our best chemists. We have been reproached lately with regarding medicine too much as the property of a clique; with resenting intrusion on our "arcana"; with being indisposed to take lessons from the uninitiated, and other like faults, of which we are entirely unconscious, and should very gladly hail the demonstration of any one definite ground upon which such charges could be substantiated. At any rate we have no secrets about chloroform: we have told the world all we know about it, and should be gratified indeed to possess one of its secrets which is declared by the public journals to be known only to the thieves. The common highwayman is an object of our scientific envy; and we should like to interrogate him by any means of physical or moral investigation which would be calculated to elicit for the benefit of mankind the marvellous secret which he is said to practise for their discomfiture and abuse. The political journals very sagely and solomnly hesitated to pronounce an opinion on the difficult question whether it might be possible, by the aid of photographic science, to obtain from the eye of a corpse a visible image of the person (say of a murderer) last impressed upon it. No doubt moonbeams extracted from cucumbers were the actual rays employed. Those journals seem, however, to have no doubt about the fact that a highwayman can, by shaking a handkerchief impregnated with chloroform under the nose of his victim, produce instantaneous insensibility. It is within the experience of medical men that anaesthesia by chloroform is not very quickly or very easily effected upon a non-consenting person, and that with the utmost resignation and good-will some five minutes or more are requisite to produce anaesthesia. . . . We can promise to the scientific thief a far more ample pecuniary reward from the honest application of his knowledge than pocket-picking or highway robbery is likely to afford, howsoever successfully pursued; and we can assure a permanent scientific reputation to his patrons and believers in the public press if they will worm out this hidden secret.

(Oct 28, 1865)

APPENDIX B

American Journal of Epidemiology
Vol. 134, No. 6
Printed in U.S.A.

Epidemiologic Patterns of Acute Diarrhea and Endemic *Shigella* Infections in Children in a Poor Periurban Setting in Santiago, Chile

Catterine Ferreccio,[1,2] Valeria Prado,[3] Alicia Ojeda,[3] Marisol Cayyazo,[2] Paulina Abrego,[2] Linda Guers,[1] and Myron M. Levine[1]

To prepare a field site for evaluating preventive interventions against endemic shigellosis, the authors followed prospectively a cohort of 360 children (90 each of children aged 0–11, 12–23, 24–35, and 36–47 months) in Santa Julia, a low socioeconomic area in Santiago, Chile, from November 1986 through April 1989 with twice weekly household visits for diarrheal disease; infants replaced children who reached 60 months of age. Coprocultures on 2 consecutive days from children with diarrhea and from age-matched controls within the cohort were cultured for *Shigella*. Bacteriologic surveillance was also maintained in the health center and children's hospital serving Santa Julia. In this community, where all households had access to potable water (68% inside) and all but 3% had access to a toilet, but where there was marked crowding, the overall incidence of diarrheal disease in the cohort was low (2.26 episodes/12 child months of observation in children aged 0–11 months and 2.09 in those aged 12–23 months), yet *Shigella* infections were common. *Shigella* accounted for 10% of diarrheal episodes in the cohort (vs. 3.2% isolation rate in controls, $p < 0.0001$). The incidence of shigellosis in children aged 12–47 months was 0.16 cases per 12 child months of observation; in the first 5 years of life, a child had a 67% chance of experiencing shigellosis. *Shigella sonnei*, *Shigella flexneri* 2a, and *S. flexneri* 6 caused > 79% of the infections. *Shigella* occurred more often in hospitalized cases of diarrhea than in age-matched cases detected in the health center or by household surveillance ($p < 0.0001$). An initial episode of *Shigella* diarrhea did not diminish overall the risk of subsequent shigellosis but did confer 72% protection ($p = 0.05$) against illness due to the homologous serotype. The high rate of both *S. sonnei* and *S. flexneri* shigellosis in a population with a low background rate of diarrhea makes Santa Julia an appropriate site for assessing the efficacy and effectiveness of measures to reduce *Shigella* infections. *Am J Epidemiol* 1991;134:614–27.

diarrhea; *Shigella*

Diarrheal disease and dysentery caused by *Shigella* constitute health problems in industrialized as well as in less-developed countries (1–3). In the less-developed world,

Received for publication January 22, 1991, and in final form May 2, 1991.

[1] Center for Vaccine Development, Division of Geographic Medicine, Department of Medicine, and Division of Infectious Diseases and Tropical Pediatrics, Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD.

[2] Programa para el Control de la Fiebre Tifoidea y Enfermedades Entericas, Ministry of Health, Santiago, Chile.

[3] Unidad de Microbiologia, Facultad de Medicina, Universidad de Chile, Sede Oriente, Santiago, Chile.

Reprint requests to Dr. M. M. Levine, Center for Vaccine Development, University of Maryland School of Medicine, 10 S. Pine St., Baltimore, MD 21201.

These field studies were supported by research contract DAMD-17-87-C-7022 from the US Army Medical Research and Development Command and by a grant from the Diarrhoeal Diseases Control Programme of the World Health Organization.

The authors thank Ana Claudia Pizarro for technical assistance and Dr. S. B. Formal for encouragement and helpful suggestions during the project.

Shigella accounts for approximately 8–13 percent of cases of diarrhea in children under 5 years of age in clinic-based surveys (4–9) and for approximately 4–8 percent of the episodes of diarrhea detected through household surveillance of cohorts of young children (10–14). With the increasing use of oral rehydration in national diarrheal disease control programs, deaths from acute diarrheal dehydration have markedly declined in many areas (15). One consequence has been an increase in the relative importance of certain other forms of diarrheal disease that are less affected by oral rehydration, such as dysentery (frank blood and mucus in stools) and persistent diarrhea (lasting >14 days) (16).

Since 1968, in addition to endemic *Shigella* disease, one serotype, *Shigella dysenteriae* 1, has been causing extensive outbreaks of severe disease accompanied by high case fatality in much of the developing world (17–20). The overall situation is further complicated by the widespread and increasing resistance of *Shigella* to previously useful antibiotics (such as ampicillin and trimethoprim/sulfamethoxazole) (17–23), which seriously hampers the treatment of severe shigellosis in less-developed countries.

Shigellosis is one bacterial enteric infection that persists in industrialized countries, despite the widespread availability of bacteriologically controlled water supplies and flush toilet sanitation (1, 2). The residual problem in developed settings largely derives from the fact that clinical infection can be transmitted by as few as 10 *Shigella* organisms (24) even without neutralization of gastric acid. Thus, *Shigella* is spread by direct fecal-hand-oral contact wherever personal hygiene is compromised, as among preschool children in day-care centers (25, 26), among patients in custodial institutions for the mentally impaired (27, 28), or among underprivileged preschool children residing under crowded conditions in inner cities (29).

Because *Shigella* infections constitute a refractory problem worldwide, the development of new and improved *Shigella* vaccines has been targeted by the World Health Organization as a high priority to provide an additional control measure (30–32). New *Shigella* vaccine candidates are reaching the stage of clinical trials (33–38). In order to assess their safety, efficacy, and practicality in distinct settings, promising *Shigella* vaccines will have to be evaluated in a variety of populations representing diverse ages, geographic areas, and socioeconomic levels. In Santa Julia, a periurban, low socioeconomic area located within Area Oriente (the Eastern Administrative Area) of Santiago, Chile, we undertook to study intensively and prospectively the epidemiology of *Shigella* infections in a population where crowding is severe but where potable water and some form of sanitation are available to most households. Heretofore, prospective studies of endemic shigellosis have generally involved rural or urban populations in less-developed countries living under conditions without potable water or sanitation (3, 10, 11, 16, 39) or in urban settings in industrialized countries (26). Santa Julia constitutes one appropriate site for assessing potential interventions against endemic shigellosis.

MATERIALS AND METHODS

Study objectives

The general objective of the Santa Julia field project was to provide a defined population of young children in which to conduct applied epidemiologic research of public health importance such as the evaluation of candidate *Shigella* vaccines. Specific objectives included a quantitation of the incidence of all diarrheal disease and of shigellosis by age and by season and an identification of the relative importance of the different *Shigella* serotypes. To accomplish these objectives, we initiated a longitudinal, prospective, community-based study that utilized household visits to detect mild diarrheal illnesses. To complement the information provided by household surveillance and to investigate more severe forms of diarrheal illness, a surveillance system for diarrheal disease was also implemented in the

local health center (Consultorio Santa Julia) and in the pediatric hospital (Hospital Calvo Mackenna) that serve this population.

Study site

Santa Julia is a periurban neighborhood of low socioeconomic level containing 133,909 inhabitants living in mostly ramshackle housing in a geographic area of 12.3 km^2. The birthrate is 19.8 per thousand, and 31 percent of the population is under 15 years of age. A total of 15,525 children less than 5 years of age resided in Santa Julia at the initiation of the study including 2,844 less than 12 months, 2,811 from 12 to 23 months, and 9,870 from 2 to 5 years of age. The Chilean National Health Service provides health care to approximately 96 percent of the population of Santa Julia through a neighborhood health center, Consultorio Santa Julia. The out-migration rate from this community is approximately 8 percent per year and largely involves relocation of residents elsewhere within metropolitan Santiago. The climate is temperate with a mild winter and a warm, rainless summer.

Study subjects

Participants were a stratified sample consisting of 360 Santa Julia children under 5 years of age, 90 falling within each one of four age strata: less than 12 months, 12–23 months, 24–35 months, and 36–47 months of age. When children reached 60 months of age, they were dropped from the study and replaced with children less than 12 months of age.

Participation, which was restricted to pediatric subjects without chronic disease or serious congenital malformations, was at the discretion of parents from whom informed consent was obtained. The protocol was approved by the Ministry of Health of Chile and by ethics committees at the University of Maryland School of Medicine, the World Health Organization, and the US Department of Defense.

Sampling procedure

The population sample was obtained by stratified sampling. Demographic information on every newborn in the community of Santa Julia is contained on a card kept within the Consultorio Santa Julia. These cards are organized according to the three geographic subsectors (of approximately equal population size) that comprise the community of Santa Julia, including Las Torres, Jaime Eyzaguirre, and Chacarillas. The children of each sector were line listed by date of birth. Children falling within the age groups 0–11 months, 12–23 months, 24–35 months, and 36–47 months (the age groups of interest) were consecutively numbered within each age group. From each sector we needed a total of 120 children equally divided among these four age groups (i.e., 30 children per group). Therefore, the number of children in each age stratum of each sector was divided by 30, thereby providing the sampling interval. The first child's card was randomly chosen; the remaining 29 cards were selected by adding the sampling interval to the number of the first card chosen. This sampling procedure was performed for each of the four age groups in each of the three sectors. There is a delay of some weeks until a card containing the demographic information on a newborn is prepared and inserted into the consultorio data base. For this reason, infants in the age group 0–2 months are underrepresented in this universe of cards from which we sampled.

Mothers of selected children were visited to explain the study and to elicit informed consent for their participation. The acceptance rate was approximately 90 percent among mothers of children in all age groups and sections. Infants who replaced children who graduated from the cohort were selected by the stratified sampling procedure described above.

Surveillance for diarrhea in the cohort

Upon inclusion into the cohort, the nutritional status of each child was recorded as expected weight for age (40). The household

of each participating child was visited twice weekly by a trained public health nurse or nurse auxiliary who interviewed the caretaker in order to elicit information about the occurrence of diarrheal illness. Systematic queries focused on the number, consistency, and character (i.e., watery, loose, bloody) of stools that occurred during each 24-hour period since the previous visit; responses were recorded using a precoded questionnaire. Associated symptoms (e.g., lethargy and vomiting) as well as objective signs of dehydration were noted, and the rectal temperature was recorded.

Oral glucose-electrolytes rehydration solution was offered by the nurse when appropriate. Criteria for a child to be seen by the study pediatrician included signs of dehydration, high fever, marked lethargy, or overt dysentery (blood in stools). Children with dysentery or with persistent diarrhea (>14 days) were treated with oral trimethoprim/sulfamethoxazole (41).

Surveillance for cases of diarrheal disease in the consultorio and in the hospital

To accomplish surveillance in the Consultorio Santa Julia (which was open Monday to Friday), a health auxiliary recorded the visits of every child less than 60 months of age with a chief complaint of diarrheal illness. The clinician caring for the child completed a summary of the clinical illness that included a description of the number and type of stools, presence of fever and vomiting, degree of dehydration (if any), and whether antibiotics were used. A single coproculture was obtained from every child with diarrheal illness seen at the consultorio.

To accomplish hospital surveillance, each day (except Saturday and Sunday) a nurse from the team visited Calvo MacKenna Children's Hospital to review all hospital admissions due to diarrheal illness in all services including emergency room, infectious diseases unit, and infant/toddlers ward. Every child who came from the community of Santa Julia was cultured (once, as described), and clinical data were systematically recorded until discharge.

Clinical definitions

Diarrhea is defined as an overt change in the child's normal stool pattern, characterized by an increase in the frequency (to at least three stools per 24-hour period) and a decrease in the consistency of stools to an unformed state. *Dysentery* refers to loose stools that contain gross blood, with or without mucus.

An *episode of diarrhea or dysentery* is considered to have commenced after 7 consecutive days without diarrhea and to have ended on the first day that was followed by 7 consecutive days without diarrhea. *Episodes of Shigella diarrhea or dysentery* are defined as above but accompanied by the isolation of *Shigella* from coprocultures taken at the time of illness. *Asymptomatic Shigella infection* refers to isolation of *Shigella* from a child in the absence of diarrheal illness.

Selection of control children

After the cohort had been assembled, the 30 children within each sector and age stratum were separated by sex and again line listed. One by one, each male on the list was matched by simple random method with another male in his group as a "one-way" control. For example, in the process of selecting controls, child 2 could not be a control for child 8 if child 8 was already selected to be the control for child 2. This process of selection of controls was repeated for the females in the group in each age stratum and sector.

Clinical specimens

When an episode of diarrhea or dysentery was detected, stool specimens or rectal swabs were obtained for bacteriologic culture on 2 consecutive days from the ill child; analogous specimens for culture were also obtained on the same 2 consecutive days from a predetermined, age-matched asymptomatic control child in the cohort.

Weekly prevalence of *Shigella*

A subsample of 120 children were cultured weekly for *Shigella*. They were selected by random sampling of 10 children from each age stratum within each of the three subsectors (total = 120 children). This bacteriologic surveillance was instituted to provide information on the magnitude of the reservoir of *Shigella* throughout the calendar year. When a child in the subsample graduated from the cohort, he was replaced with another randomly selected child in order to maintain circa 120 prevalence cultures per week.

Laboratory methods

Stool samples were transported in glycerol phosphate-buffered saline (42) and inoculated onto plates of MacConkey's, xylose lysine desoxycholate, and *Salmonella-Shigella* agar (42). Plates were incubated at 35–37°C for 18–24 hours. Suspicious colonies were subcultured to slants of Kligler's iron agar. Those giving a typical *Shigella* pattern were confirmed by standard biochemical tests and serogrouped by agglutination with specific antisera (43). Serotyping of *Shigella flexneri* isolates was performed by the method of Carlin et al. (44) using monoclonal antibodies kindly provided by Nils Carlin of the Swedish Bacteriological Laboratory and Alf Lindberg of Huddinge Hospital, Stockholm, Sweden.

Assessment of sociodemographic factors and levels of sanitation and water supply

Information on sociodemographic characteristics, water quality, sanitation, and hygiene levels was obtained at baseline through a questionnaire that focused on information about the mother's education, the occupation of the head of the household, the type of housing and ownership, the degree of household crowding, the type of water supply and waste disposal, and the presence of selected possessions (e.g., a refrigerator). The density of houseflies was not quantitated.

Epidemiologic measures

The mean incidence of diarrheal or dysenteric episodes per child per 12 months of observation in each age group was calculated by dividing the total number of episodes detected by the total child months of observation, for children within that age group, and multiplying by 12. The analogous incidence rates for each age group were calculated for *Shigella*-positive diarrhea and dysentery. To calculate the cumulative percentage of children who experienced diarrhea in 1 year, the number of children within each age bracket who were observed for at least 12 consecutive months and who experienced at least one bout of diarrhea was divided by the total number of children of that age followed for at least 12 consecutive months and expressed as percentage.

Statistical methods

Rates were compared by χ^2 or Fisher's exact test (two tails). The Wilcoxon rank sum test was used where a nonparametric test was indicated.

RESULTS

Characteristics of the Santa Julia participants

Between November 1, 1986, and April 30, 1989, 504 children entered the study, 249 males and 255 females; only two children selected by the sampling method were ineligible due to chronic disease (Down's syndrome and cerebral palsy). A total of 360 children were in the cohort at the beginning of the study, while at study termination the cohort numbered 306. Despite living in substandard housing and otherwise representing a pediatric population of low socioeconomic level, 95 percent of the children were well nourished; only 5 percent suffered from mild (first degree) malnutrition, while just a single child presented moderate malnutrition. The median duration of breast feeding was 7 months, ranging from 0 to 48 months.

Baseline sociodemographic information

The median family size was six, and the majority of heads of household held no jobs or worked only sporadically. Sixty-two percent of the families lived in houses that they neither owned nor rented. Extremely crowded living conditions were the general rule: 58 percent lived in cramped dwellings in which the number of beds was less than the number of household occupants minus the number of couples. All dwellings had access to potable water, although 32 percent of families had to go outside the home to collect water. All households had garbage removal twice each week. Sanitary facilities for disposing of human waste were quite variable: 64 percent had a toilet inside the home, 34 percent had access to toilets outside the home, and 3 percent of families had only latrines. Less than one-half of the families (48 percent) had a refrigerator. Two percent of mothers had no formal education. Twenty percent of mothers work outside the home; in general, the children of working mothers are cared for by an older sibling or a grandmother.

Retention of the cohort

During the 30 months that the study lasted, the cohort was followed prospectively with an average observation period of 20 months per child (range, 1–30 months). Of the total of 504 children who entered the study during these 2½ years, 82 (16.2 percent) left before study termination, and one died before the end of the study. Sixty-seven of the 82 children lost to follow-up were the consequence of migration out of the study area, while only 15 of 504 children (3.0 percent) dropped out because of refusal to continue participation.

This pediatric cohort provided 9,951 child months of observation, equivalent to 332 children observed during 30 months (table 1). Ten percent of this total observation was contributed by infants under 12 months of age and 18 percent by children aged 12–23 months; 72 percent of the total observation was provided by children aged 24–35, 36–47, or 48–59 months of age (table 1).

Incidence and seasonality of diarrhea

A total of 1,218 episodes of diarrhea were detected in the cohort through household surveillance. In 1,137 (93.3 percent) of these episodes, stool samples were obtained from the cases and, in 1,129 (92.6 percent) of the episodes, stool samples were obtained from both the cases and the matched controls.

The incidence of diarrhea showed a marked seasonality in all age groups with significantly higher rates being recorded in the warm summer months of December through February (866 episodes in 5,947 summer months of observation) than in the cool winter months of June through August (352 episodes per 4,004 winter months of observation) ($p = 0.000001$).

As summarized in table 1, all measures of diarrheal disease were highest in infants less than 12 months of age and decreased thereafter in the older age groups. These age differences remained when corrected for season.

Hospitalization

Over the course of the study period (30 months), 257 of the 15,525 children less than 5 years of age in Santa Julia had a total of 280 hospitalizations due to diarrhea, giving an annual rate of 7.2 hospitalizations due to diarrheal disease per 1,000 children under 5 years of age. Rates of hospitalization decreased with age from 30.5 hospitalizations per 1,000 infants under 12 months to 0.2 hospitalizations per 1,000 children over 47 months of age.

Seven children (1.4 percent) of the 504 who participated in the cohort study over the 30 months of surveillance were hospitalized because of acute diarrheal illness during the surveillance period, giving a yearly rate of 8.4 hospitalizations per 1,000 children under 5 years of age; age-specific hospitalization rates ranged from 62.5 per 1,000 infants under 12 months of age to 0.0 per 1,000 children above 47 months of age.

Clinical features of cases detected through the three tiers of surveillance

Clinical features of the cases of diarrheal disease detected through the three tiers of

TABLE 1. Incidence of diarrhea by age and percentage of children followed at least 1 year who developed diarrhea during prospective surveillance of a cohort of children: Santa Julia, Santiago, Chile, November 1986 through April 1989

Age group (months)	No. of children by age at admission to cohort	Total child months of observation	Total no. of episodes of diarrhea	Incidence of all episodes/12 months	% of children who experienced diarrhea in 1 year
0–11	219	959	181	2.26	79
12–23	95	1,760	306	2.09	64
24–35	99	2,360	287	1.46	61
36–47	91	2,469	264	1.28	56
≥48	0	2,403	180	0.90	50
Total	504	9,951	1,218	1.47	

TABLE 2. Comparison of clinical features of cases of diarrhea seen at the three levels of surveillance: Santa Julia, Santiago, Chile, November 1986 through April 1989

Site of surveillance	Total no. of diarrheal cases	% <12 months of age	Male: female ratio	% with fever	% with dysentery	% with dehydration
Hospital	290	77 (a)*	1.35	73 (b)	21 (c)	85 (d)
Consultorio	1,655	37 (e)	1.10	40 (f)	9 (g)	11 (h)
Household surveillance	1,218	15 (i)	0.90	36 (j)	7 (k)	3 (l)

* a vs. e, $p < 0.00001$; b vs. f, $p < 0.00001$; c vs. g, $p < 0.0001$; d vs. h, $p < 0.0001$; e vs. i, $p < 0.00001$; f vs. j, $p = 0.031$; g vs. k, p = not significant; h vs. l, $p < 0.00001$; a vs. i, $p < 0.00001$; b vs. j, $p < 0.00001$; c vs. k, $p < 0.0001$; d vs. l, $p < 0.00001$.

surveillance are summarized in table 2 where a clear-cut gradient is seen with hospitalized cases being the most severely ill but also the youngest.

Duration of diarrhea

The average duration of diarrheal illness detected by household surveillance was 7.3 days. Twenty-four percent of episodes lasted less than 4 days, and 40 percent had durations between 4 and 7 days; 10.1 percent of episodes continued for more than 14 days to reach the criterion for persistent diarrhea (16). There was no difference in duration by age.

Isolation of *Shigella* in cases and in matched controls

Overall, *Shigella* was isolated in 10.0 percent of the 1,137 cultured episodes of diarrheal illness in the cohort under prospective household surveillance but from only 3.2 percent of 1,126 matched controls without diarrhea (table 3), a highly significant difference ($p < 0.0000001$). In every age group except infants less than 12 months, the isolation rate of *Shigella* was significantly higher in cases than in controls. The relative risk was highest in the second and third years of life.

TABLE 3. Isolation of *Shigella* from diarrheal cases and controls, by age, in a cohort of children under active household surveillance: Santa Julia, Santiago, Chile, November 1986 through April 1989

Age group (months)	Clinical status	Diarrheal episodes	Episodes with *Shigella*		p value	RR*
			No.	%		
0–11	Diarrhea	171	8	4.7	0.4	2.0
	Controls	167	4	2.4		
12–23	Diarrhea	287	23	8.0	0.00014	7.3
	Controls	285	3	1.0		
24–35	Diarrhea	274	30	10.9	0.00043	3.8
	Controls	273	8	2.9		
36–47	Diarrhea	245	35	14.3	0.0053	2.3
	Controls	242	15	8.2		
≥48	Diarrhea	160	18	11.3	0.029	3.0
	Controls	159	6	3.8		
Totals	Diarrhea	1,137	114	10.0	<0.000001	3.1
	Controls	1,126	36	3.2		

* RR, relative risk.

Age-specific incidence of *Shigella* diarrhea and dysentery

Rates of shigellosis were lowest in infants less than 1[illegible] months of age and in children over 4 yea[illegible] of age; the incidence in children 12–47 mo[illegible]hs of age was impressively consistent, ranging from 0.15 to 0.17 episodes per 12 child months of observation (table 4). Overall, approximately one-fourth of the cases of *Shigella* diarrheal infection (27 of 114 cases, 23.7 percent) manifested overt dysentery. The data in table 4 suggest that, during the first 5 years of life, a child in Santa Julia has a 67 percent likelihood of experiencing an episode of diarrheal illness due to *Shigella* and a 15 percent chance of having *Shigella* dysentery.

Seasonality of *Shigella* infections

The isolation of *Shigella* showed a marked seasonality with a notable increase in the warm months of the year (figure 1). Ninety-six (84.2 percent) of the 114 isolations of *Shigella* in the cohort occurred during the 5,947 summer child months of observation versus only 18 isolations during the 4,004 winter child months of observation ($p < 0.000001$).

Rate of isolation of *Shigella* in relation to surveillance method, sampling site, and clinical syndrome

The rate of isolation of *Shigella* by age varied in relation to the site of sampling (home, active surveillance; consultorio or hospital, passive surveillance) (table 5). Based on data from single coprocultures (in order to standardize methods used at the different sites), a gradient was observed in the rate of isolation of *Shigella*, being highest in hospitalized cases and lowest in cases detected during household visits. In age groups 12–23 and 24–35 months, these differences were highly significant (table 5). The rate of isolation from cases of overt dysentery (31.5 percent) was more than four times higher than that from cases of non-dysenteric illness (7.4 percent) ($p < 0.000001$) (table 6).

Serotypes of *Shigella* in Santa Julia

Three serotypes, *Shigella sonnei*, *S. flexneri* 2a, and *S. flexneri* 6, accounted for 79.3 percent of the cases of shigellosis in Santa Julia detected through household surveillance and 85.7 percent of the cases detected at treatment facilities. *S. flexneri* was found more often in hospitalized children than in cases of diarrhea detected otherwise (table 7).

Clinical features of shigellosis

The median age of symptomatic children from whom *Shigella* was isolated was 34 months (range, 1–65 months), while the median age of culture-positive asymptomatic children was 40 months (range, 9–60 months) (p = not significant, Wilcoxon rank sum). A total of 53 of 114 symptomatic children (46.5 percent) with *Shigella* infection detected through household visits had fever; 34 percent had vomiting; 27 percent had dysentery. The mean duration of diarrhea was 9 days (range, 1–48 days).

TABLE 4. Number of episodes and incidence of all *Shigella* diarrheal illness, nondysenteric *Shigella* diarrhea, and *Shigella* dysentery in a cohort of children followed prospectively: Santa Julia, Santiago, Chile, November 1986 through April 1989

Age group (months)	Child months of observation	Episodes of *Shigella* diarrheal illness					
		All clinical types		Nondysenteric diarrhea		Dysentery	
		No.	Incidence/12 child months	No.	Incidence/12 child months	No.	Incidence/12 child months
0–11	959	8	0.10	8	0.1	0	0.0
12–23	1,760	23	0.16	16	0.1	7	0.05
24–35	2,360	30	0.15	21	0.1	9	0.05
36–47	2,469	35	0.17	28	0.1	7	0.03
≥48	2,403	18	0.09	14	0.07	4	0.02

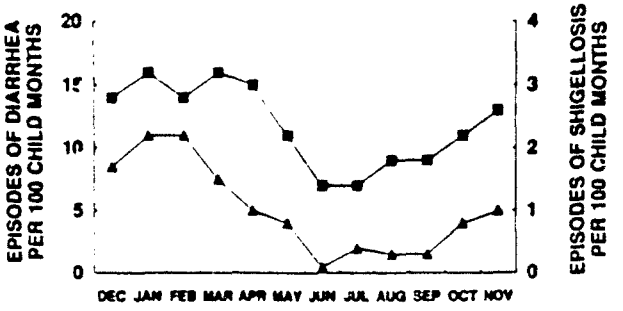

FIGURE 1. The monthly variation in the incidence of diarrheal disease (■) and of shigellosis (▲), expressed as episodes per 100 child months of observation, is shown for a cohort of children <60 months of age in Santa Julia, Santiago, Chile, who were followed for 30 consecutive months between November 1986 and April 1989 with twice weekly household visits to detect episodes of diarrhea. The incidences of both diarrhea and shigellosis were highest in the summer months of December through February.

TABLE 5. A comparison of the relative frequency of isolation of *Shigella* by sampling site and by age group: Santa Julia, Santiago, Chile, November 1986 through April 1989

Sampling site	Age group (months)	No. of diarrheal episodes cultured†	No. of episodes due to *Shigella*
Active surveillance			
Cohort	0–11	171	6 (3.5)‡ (a)*
	12–23	287	19 (6.6) (b)
	24–35	274	23 (8.4) (c)
	36–47	245	31 (12.7)
	≥48	160	13 (8.1)
Passive surveillance			
Consultorio	0–11	605	30 (5.0) (d)
	12–23	585	72 (12.3) (e)
	24–35	229	36 (15.7) (f)
	36–47	119	18 (15.1)
	≥48	117	12 (10.3)
Hospital	0–11	215	17 (7.9) (g)
	12–23	39	12 (30.8) (h)
	24–35	19	7 (36.8) (i)
	36–47	5	0 (0.0)
	≥48	2	2 (100.0)

* a vs. d, $p = 0.55$; b vs. e, $p = 0.014$; c vs. f, $p = 0.016$; d vs. e, $p = 0.15$; e vs. h, $p = 0.0025$; f vs. i, $p = 0.043$; a vs. g, $p = 0.11$; b vs. h, $p = 0.000006$; c vs. i, $p = 0.0036$.

† Only one culture per child per episode was obtained for children seen at the consultorio and at the hospital. Therefore, for purposes of comparison, only the first culture from the active surveillance cohort was included in the analysis.

‡ Numbers in parentheses, percentage.

Reinfections by *Shigella*

A total of 85 children in the cohort experienced just a single episode of diarrhea in which *Shigella* was isolated, while 14 others suffered from more than one bout of shigellosis (13 children experienced two separate episodes of *Shigella* diarrheal illness and one child had three). The specific serotypes were determined in the repeat infections suffered by 13 of the 14 children: the remaining child experienced two episodes of diarrhea due to *Shigella boydii*, and the *boydii* isolates were not serotyped. In only three of the 13 instances were repeat infections due to the same serotype: two children each had repeat *S. sonnei* infections, while one other child had shigellosis twice due to *S. flexneri* 2a. One of the two children who had repeat *sonnei* diarrheal infections subsequently de-

TABLE 6. Comparison of the isolation rate of *Shigella* from cases of diarrhea versus cases of dysentery at three sampling sites: Santa Julia, Santiago, Chile, November 1986 through April 1989

Site of sampling	Nondysenteric diarrhea				Dysentery		
	Total cases	No. studied*	No. with *Shigella*	%	Total cases	No. with *Shigella*	%
Active surveillance							
Cohort	1,126	1,048†	68	6.5	89	24†	27.0
Passive surveillance							
Consultorio	1,541	1,508	124	8.2	144	44	30.6
Hospital	221	220	14	6.4	59	24	40.7
Total	2,888	2,774	206	7.4	292	92	31.5

* A coproculture was obtained.
† For purposes of comparison, only the first stool sample was considered, although in this group two cultures were obtained on consecutive days.

TABLE 7. Serogroups of *Shigella* and serotypes of *Shigella flexneri among isolates obtained from different sampling sites: Santa Julia, Santiago, Chile, November 1986 through April 1989**

Site of sampling	*Shigella sonnei*	*Shigella boydii*	*Shigella dysenteriae*	*Shigella flexneri*	*S. flexneri* serotypes							
					1a	1b	2a	2b	3a	3b	6	1b + 2a
Active surveillance												
Cohort by age group (months)												
0–11	6	1	0	1	0	0	0	0	0	0	1	
12–23	13	2	0	8	0	2	4	0	0	1	1	
24–35	11	3	1	15	0	1	8	0	0	0	3	1
36–47	12	2	0	21	0	2	9	2	3	1	4	
≥48	5	1	0	12	0	0	4	0	2	0	6	
Total	47 (41)††	9 (7.9)	1 (0.9)	57 (50)	0	5	25	2	5	2	15	
Passive surveillance												
Consultorio by age group (months)												
0–11	9	2	0	19	0	1	8	0	2	0	6	
12–23	36	1	1	34	1	5	11	0	0	0	10	
24–35	19	3	0	14	0	3	10	0	0	0	1	
36–47	10	1	0	7	0	0	4	0	0	0	3	
≥48	6	0	0	6	0	0	4	0	1	0	1	
Total	80 (47.6)	7 (4.2)	1 (0.6)	80 (47.6)	1	9	37	0	3	0	21	
Hospital by age group (months)												
0–11	6	0	0	11	0	2	7	0	0	0	1	
12–23	2	0	0	10	1	0	6	0	0	0	2	
24–35	2	0	0	5	0	2	0	0	1	0	2	
36–47	0	0	0	0	0	0	0	0	0	0	0	
≥48	0	0	0	2	0	0	2	0	0	0	0	
Total	10 (26.3)	0	0	28 (73.7)	1	4	15	0	1	0	5	

* Isolates from 152 of the 165 children infected with *S. flexneri* (92%) were available for serotyping.
† Numbers in parentheses, percentage of isolates.

veloped diarrhea due to *S. boydii*. In the other 10 children with repeat infections, the second episode of *Shigella* diarrhea was due to a different serogroup.

The serotyping data proved extremely helpful in allowing us to examine infection-derived immunity. A total of 99 children experienced an initial episode of shigellosis during 8,381 child months of observation for a rate of 1.2 infections per 100 child

months. Since three serotypes, *S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 6, accounted for 78 of these initial episodes (79 percent) during 8,609 child months of observation (0.96 episodes/100 child months), we attempted to quantitate the degree of homologous and heterologous immunity that is conferred by an initial clinical infection due to *Shigella* by concentrating on these three common serotypes. Over a further 1,200 child months of observation thereafter, 10 of these 78 children experienced a second episode of shigellosis (0.83 episodes/100 child months) due to one of these three serotypes. Thus, an initial episode of shigellosis due to *S. sonnei*, *S. flexneri* 2a, or *S. flexneri* 6 conferred only 13.5 percent protection overall against subsequent shigellosis due to one of these three serotypes ($p = 0.93$). Nevertheless, among these 10 children who suffered a second clinical infection, repeat infections due to the identical serotype in the same child occurred only three times during 1,279 months of further observation (0.23 episodes/100 child months). This serotype-specific reinfection rate (0.23/100) is significantly lower than the rate for second shigellosis episodes overall due to these three serotypes (0.83 episodes/100 child months) ($p = 0.05$) and represents 72 percent serotype-specific protection against repeat shigellosis.

Weekly prevalence cultures and the magnitude of the reservoir of *Shigella*

During the 30-month study, a total of 12,622 stool samples were obtained from the children who were routinely sampled each week in the field, representing 12,622 child weeks of bacteriologic surveillance for carriage of *Shigella*. *Shigella* was prevalent during 1.80 percent of the total 12,622 child weeks of observation; excretion was subclinical during 1.56 percent of the child weeks of observation. Seventy-seven (49 percent) of the 158 children who contributed these weekly coprocultures excreted *Shigella* on at least one occasion.

During the total 120 calendar weeks of surveillance of this subcohort, one or more children had positive cultures for *Shigella* during 99 (82.5 percent) of the weeks, demonstrating that a detectable reservoir of *Shigella* was present within the pediatric population in Santa Julia during most of the calendar year. A definite seasonality was observed. From November to April, the rate of isolation of *Shigella* was 2.5 per 100 surveillance coprocultures, while it was 0.97 per 100 cultures from May to October ($p = 0.000001$).

DISCUSSION

Surveillance for diarrheal disease and for shigellosis was initiated to prepare a site where preventive measures against diarrheal diseases and *Shigella*, in particular, could be evaluated in pediatric populations at risk under conditions of natural transmission. While Santa Julia is an underprivileged periurban community characterized by marked crowding, it is nevertheless notably more developed than most other communities in developing countries where pediatric cohorts have been prospectively studied to quantitate the incidence of diarrheal disease (3, 10–14, 16, 45). For example, all households had access to bacteriologically monitored, treated water supplies (although 32 percent had to go outside to collect the water), and two-thirds had a flush toilet within the dwelling. Based on these factors, one might expect the overall incidence of diarrheal disease in young children to be lower than in analogous studies that have been carried out elsewhere in developing countries. Caution must be exercised in comparing results of longitudinal cohort studies of diarrheal disease because of differences in methodology (e.g., in definitions used, in the frequency of household surveillance visits, and in age composition and size of the cohort). Nevertheless, the rates of 2.26 episodes of diarrhea per 12 child months of observation among infants and of 2.09 episodes per 12 child months of observation among children aged 12–23 months in Santa Julia are notably less than the 4–8 episodes per 12 child months

recorded in children of the same age elsewhere in Latin America, including a "favella" in Fortaleza, Brazil (10), a "pueblo nuevo" in Lima, Peru (45), a Mayan highland village in Guatemala (3), and a village in Mexico (12). Only the rural cohort in Puriscal, Costa Rica, of Vives et al. (46), the well-to-do urban population followed by Guerrant et al. (10) in Fortaleza, and an urban cohort followed (once weekly) in Buenos Aires (47) show comparable low incidence rates during the first few years of life. These data support the expectation that provision of potable water and means of disposing of human waste decrease the transmission of many enteric pathogens, resulting in lower diarrhea rates in young children.

Shigella differs from many other bacterial enteropathogens in that its transmission is more closely correlated with practices of hygiene than with levels of sanitation; thus, shigellosis can remain endemic in the face of modern sanitation if hygiene is compromised. In this regard, the marked crowding characteristic of Santa Julia fosters conditions compatible with the transmission of *Shigella*. One might therefore expect shigellosis to be endemic in Santa Julia, despite the low overall incidence of diarrhea in children. The prospective surveillance data confirm this. Approximately 10 percent of episodes of diarrhea in young children in Santa Julia detected by household visits are associated with *Shigella*, while in summer months the figure rises to circa 20 percent of cases. Indeed, during the first 5 years of life in Santa Julia, a child has approximately a 67 percent chance of experiencing a clinical illness due to *Shigella* (table 4).

The prominent rates of *Shigella* infection in Santa Julia, the use of three tiers of prospective bacteriologic surveillance (household, consultorio, and hospital), and the serotyping of *S. flexneri* isolates provided an opportunity to investigate certain aspects of the epidemiology of *Shigella* infections that have been alluded to in the past but that generally have not been well documented. These include the relative importance of different serotypes and the association of *Shigella* with distinct clinical syndromes of diarrheal illness. In this study, we confirm that *Shigella* is much more frequently isolated from cases of overt dysentery than from cases of diarrhea and show that *Shigella* has a propensity to cause severe diarrheal illness (its isolation rate paralleled the severity of cases, being highest in hospitalized children).

This prospective study shows that only a few serotypes (*S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 6) account for a large proportion (>79 percent) of the cases and implies that an efficacious vaccine directed against just a few serotypes could have a notable impact in diminishing *Shigella* disease. In Santa Julia, as in most areas of the developing world, *S. flexneri* 2a is the most prevalent *flexneri* serotype (3, 7, 14, 39, 48, 49).

S. dysenteriae and *S. flexneri* are common in less-developed countries (2, 7, 50), while *S. sonnei*, by far the major serotype found in industrialized countries (1, 51), is relatively uncommon in situations of underdevelopment (2, 7, 50). Enigmatically, despite the relative paucity of *S. sonnei* disease among indigenous persons living in less-developed countries (7, 50–52), *S. sonnei* commonly causes shigellosis among travelers to these same less-developed areas (52–54). It is thus of interest that Santa Julia shows a somewhat unusual, intermediate pattern wherein both *S. sonnei* and *S. flexneri* isolates are common. This likely reflects a community that is undergoing active development, transforming from a less-developed toward an industrialized situation (51). Paradoxically, the universal availability of monitored, potable water in Santa Julia may account for the high frequency of *S. sonnei* infections. The possible explanation is that some strains of *Plesiomonas shigelloides*, an autochthonous bacterial species of surface waters (55), express a polysaccharide O antigen identical to that of *S. sonnei* (56). Under less-developed conditions, repeated ingestion of *Plesiomonas* bacteria through consumption of untreated surface waters may stimulate cross-protection against *S. sonnei*, since O antibodies are believed to mediate protection (57). Whatever the explanation, the unexpectedly frequent occur-

rence of *S. sonnei* infections among children in Santa Julia makes this a particularly interesting site because the efficacy and effectiveness of interventions can be measured against *S. sonnei* as well as against *S. flexneri*.

This prospective study also provides some insights into the acquisition of immunity to *Shigella* in that prior infection with one serotype of *Shigella* appeared to protect against subsequent clinical infection with the identical serotype but not against diarrheal illness due to other serotypes. Formal et al. (58) have recently documented this lack of cross-protection in monkeys. Monkeys that were experimentally infected with *S. flexneri* 2a were completely protected against homologous challenge 5 weeks later with *S. flexneri* 2a but not against challenge with *S. sonnei*.

Shigella vaccine candidates of different varieties are reaching the stage of clinical testing (31- 38). Santa Julia offers an unusually attractive site to undertake evaluations of preventive measures against *Shigella* infections because of its relatively high rate of seasonal, endemic shigellosis and dysentery in a background where the overall incidence of diarrhea is not high.

REFERENCES

1. Blaser MJ, Pollard RA, Feldman RA. *Shigella* infections in the United States, 1974–1980. J Infect Dis 1983;147:771–5.
2. Khan MU, Roy NC, Islam MR, et al. Fourteen years of shigellosis in Dhaka: an epidemiological analysis. Int J Epidemiol 1985;14:607–13.
3. Mata LJ. The children of Santa Maria Cauque: a prospective field study of health and growth. Cambridge, MA: MIT Press, 1978.
4. Stoll B, Glass RI, Huq MI, et al. Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. BMJ 1982;285:1185–8.
5. Taylor DN, Echeverria P, Pal T, et al. The role of *Shigella* spp., enteroinvasive *Escherichia coli*, and other enteropathogens as causes of childhood dysentery in Thailand. J Infect Dis 1986;153:1132–8.
6. Echeverria P, Seriwatana J, Taylor DN, et al. A comparative study of enterotoxigenic *Escherichia coli*, *Shigella*, *Aeromonas*, and *Vibrio* as etiologies of diarrhea in northeastern Thailand. Am J Trop Med Hyg 1985;34:547–54.
7. Casalino M, Yusuf MW, Nicoletti M, et al. A two year study of enteric infections associated with diarrhoeal diseases in children in urban Somalia. Trans R Soc Trop Med Hyg 1988;82:637–41.
8. Mata L, Simhon A, Padilla R, et al. Diarrhea associated with rotaviruses, enterotoxigenic *Escherichia coli*, *Campylobacter*, and other agents in Costa Rican children, 1976–1981. Am J Trop Med Hyg 1986;32:146–53.
9. Black RE, Merson MH, Rahman ASM, et al. A two year study of bacterial, viral, and parasitic agents associated with diarrhea in rural Bangladesh. J Infect Dis 1980;142:660–4.
10. Guerrant RL, Kirchoff LV, Shields DS, et al. Prospective study of diarrheal illnesses in notheastern Brazil: patterns of disease, nutritional impact, etiologies, and risk factors. J Infect Dis 1981;148: 986–97.
11. Black RE, Brown KH, Becker S, et al. Longitudinal studies of infectious diseases and physical growth of children in rural Bangladesh. II. Incidence of diarrhea and association with known pathogens. Am J Epidemiol 1982;115:315–24.
12. Cravioto A, Reyes RE, Ortega R, et al. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated-pathogens during the first two years of life. Epidemiol Infect 1988;101:123–34.
13. Zaki AM, DuPont HL, El-Alamy MA, et al. The detection of enteropathogens in acute diarrhea in a family cohort population in Egypt. Am J Trop Med Hyg 1986;35:1013–22.
14. Joe LK, Rukmono B, Oemijati S, et al. Diarrhoea among infants in a crowded area of Djakarta, Indonesia. A longitudinal study from birth to two years. Bull World Health Organ 1966;34:197–210.
15. El-Rafie M, Hassouna WA, Hirschhorn N, et al. Effect of diarrhoeal disease control on infant and childhood mortality in Egypt. Report from the National Control of Diarrheal Diseases Project. Lancet 1990;335:334–8.
16. Bhan MK, Bhandari N, Sazawal S, et al. Descriptive epidemiology of persistent diarrhoea among young children in rural northern India. Bull World Health Organ 1989;67:281–8.
17. Mata LJ, Gangarosa EJ, Caceres A, et al. Epidemic Shiga bacillus dysentery in Central America. I. Etiologic investigations in Guatemala, 1969. J Infect Dis 1970;122:170–80.
18. Rahaman MM, Khan MM, Aziz KMS, et al. An outbreak of dysentery caused by *Shigella dysenteriae* type 1 on a coral island in the Bay of Bengal. J Infect Dis 1975;132:15–19.
19. Erbright JR, Moore EC, Sanborn WR, et al. Epidemic Shiga bacillus dysentery in central Africa. Am J Trop Med Hyg 1984;33:1192–7.
20. Taylor DN, Bodhidatta L, Brown E, et al. Introducion and spread of multi-resistant *Shigella dysenteriae* 1 in Thailand. Am J Trop Med Hyg 1989;40:77–85.
21. Macaden R, Gokul BN, Pereira P, et al. Bacillary dysentery due to multidrug-resistant *Shigella dysenteriae* type 1. Indian J Med Res 1980;71: 178–85.
22. Frost JA, Rowe B, Vandepitte J, et al. Plasmid characterisation in the investigation of an epidemic caused by multiply resistant *Shigella dysenteriae* type 1 in central Africa. Lancet 1981;2:1074–6.

23. Macaden R, Bhat P. The changing pattern of resistance to ampicillin and co-trimoxazole in *Shigella* serotypes in Bangalore, southern India. (Letter). J Infect Dis 1985;152:1348.
24. DuPont HL, Levine MM, Hornick RB, et al. Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis 1989;159:1126–8.
25. Weissman JB, Gangarosa EJ, Schmerler A, et al. Shigellosis in day care centers. Lancet 1975;1: 88–90.
26. Pickering L, Evans DG, DuPont HL, et al. Diarrhea caused by *Shigella*, rotavirus, and *Giardia* in day care centers: prospective study. J Pediatr 1981; 8:539–47.
27. DuPont HL, Gangarosa EJ, Reller LB, et al. Shigellosis in custodial institutions. Am J Epidemiol 1970;92:172–9.
28. Coles FB, Kondracki SF, Gallo RJ, et al. Shigellosis outbreaks at summer camps for the mentally retarded in New York State. Am J Epidemiol 1989;130:966–75.
29. Nelson JD, Kusmiesz HT, Haltalin KC. Endemic shigellosis: a study of fifty households. Am J Epidemiol 1967;86:683–9.
30. Diarrhoeal Diseases Control Programme, World Health Organization. Biomedical and epidemiological research priorities of global scientific working groups. Geneva: WHO, 1987. (WHO/CDD/86.8 Rev. 1).
31. Levine MM. Modern vaccines. Enteric infections. Lancet 1990;1:958–6..
32. World Health Organzation. Development of vaccines against shigellosis: memorandum of a WHO meeting. Bull World Health Organ 1987;65:17–25.
33. Formal SB, Hale TL, Kapfer C, et al. Oral vaccination of monkeys with an invasive *Escherichia coli* K-12 hybrid expressing *Shigella flexneri* somatic antigen. Infect Immun1984;46:465–9.
34. Black RE, Levine MM, Clements ML, et al. Prevention of shigellosis by a *Salmonella typhi-Shigella sonnei* bivalent vaccine. J Infect Dis 1987;155:1260–5.
35. Herrington DA, Van de Verg L, Formal SB, et al. Studies in volunteers to evaluate candidate *Shigella* vaccines: further studies with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by *Shigella sonnei* disease. Vaccine 1990;8:353–7.
36. Lindberg AA, Karnell A, Stocker BAD, et al. Development of an auxotrophic oral live *Shigella flexneri* vaccine. Vaccine 1988;6:147–50.
37. Sansonetti PJ, Arondel J. Construction and evaluation of a double mutant of *Shigella flexneri* as a candidate for oral vaccination against shigellosis. Vaccine 1989;7:443–50.
38. Robbins JB, Schneerson R. Polysaccharide-protein conjugates: a new generation of vaccines. J Infect Dis 1990;161:821–32.
39. Higgins AR, Floyd TM, Kader MA. Studies in shigellosis. II. Observations on incidence and etiology of diarrheal disease in Egyptian children. Am J Trop Med Hyg 1955;4:271–80.
40. Gomez F, Galvan R, Cravioto J, et al. Malnutrition in infancy and childhood with special reference to kwashiorkor. In: Levine SZ, ed. Advances in pediatrics. Vol 8. Chicago: Year Book Medical Publishers, Inc, 1955:131–69.
41. DiJohn D, Levine MM. Treatment of diarrhea. Infect Dis Clin North Am 1988;2:719–45.
42. Morris GK, Koehler JA, Gangarosa EJ, et al. Comparison of media for direct isolation and transport of Shigellae from fecal specimens. Appl Microbiol 1970;19:434–7.
43. Kelly MT, Brenner DJ, Farmer JJ III. Enterobacteriaceae. In: Lennette EH, Balows A, Hausler WJ Jr, et al., eds. Manual of clinical microbiology. 4th ed. Washington, DC: American Society for Microbiology, 1985:263–77.
44. Carlin NA, Rahman M, Sack DA, et al. Use of monoclonal antibodies to type *Shigella flexneri* in Bangladesh. J Clin Microbiol 1989;27:1163–6.
45. Black RE, Lopez de Romaña G, Brown KH, et al. Incidence and etiology of infantile diarrhea and major routes of transmission in Huascar, Peru. Am J Epidemiol 1989;129:785–99.
46. Vives M, Mata LJ, Castro B, et al. Estudio de Puriscal. V. Infeccion enterica en ninos menores de dos anos. (In Spanish). Rev Med Hosp Nacional de Ninos (Costa Rica). 1982;17:57–69.
47. Grinstein S, Gómez JA, Bercovich JA, et al. Epidemiology of rotavirus infection and gastroenteritis in prospectively monitored Argentine families with young children. Am J Epidemiol 1989;130:300–8.
48. Olarte J, Galindo E, Formal SB. Serotypes of *Shigella flexneri* found in children in Mexico City. Bol Of Sanit Panam 1959;47:507–8.
49. Piechaud D, Szturm-Rubinstein S, D'Hauteville H, et al. Epidemiologie de la dysentérie bacillaire à Djibouti. (In French). Bull Soc Pathol Exot Filiales 1971;64:37–42.
50. Stoll B, Glass RI, Huq I, et al. Epidemiologic and clinical features of patients with *Shigella* who attended a diarrheal disease hospital in Bangladesh. J Infect Dis 1982;146:177–99.
51. Kostrzewski J, Stypulska-Misiurewics H. Changes in the epidemiology of dysentery in Poland and the situation in Europe. Arch Ther Exp (Warsz) 1968;16:429–51.
52. Berrkman E. 771 *Shigella* strains isolated in 15 years from Americans stationed in Ankara: comparison of these results with findings in Turkish citizens. Mikrobiyol Bul 1976;10:473–99.
53. Taylor DN, Houston R, Shlim DR, et al. Etiology of diarrhea among travelers and foreign residents in Nepal. JAMA 1988;260:1245–8.
54. Speelman P, Struelens MJ, Sanyal SC, et al. Detection of *Campylobacter jejuni* and other potential pathogens in travellers' diarrhoea in Bangladesh. Scand J Gastroenterol 1983;18(suppl 84):19–23.
55. Arai T, Ikejima N, Itoh T, et al. A survey of *Plesiomonas shigelloides* from aquatic environments, domestic animals, pets, and humans. J Hyg (Lond) 1980;84:203–11.
56. Basu S, Tharanathan RN, Kontrohr T, et al. Chemical structure of the lipid A component of *Plesiomonas shigelloides* and its taxonomical structure. FEMS Microbiol Lett 1985;28:7–10.
57. Cohen D, Green MS, Block C, et al. Serum antibodies to lipopolysaccharide and natural immunity to shigellosis in an Israeli military population. J Infect Dis 1988;157:1068–71.
58. Formal SB, Oaks EV, Olsen RE, et al. The effect of prior infection with virulent *S. flexneri* 2a on the resistance of monkeys to subsequent infection with *Shigella sonnei*. J Infect Dis (in press).

APPENDIX C

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1990, p. 2485–2490
0095-1137/90/112485-06$02.00/0

Vol. 28, No. 11

Practical and Economical Method for Using Biotinylated DNA Probes with Bacterial Colony Blots To Identify Diarrhea-Causing *Escherichia coli*

KAREN G. GICQUELAIS,[1] MARY M. BALDINI,[1] JUAN MARTINEZ,[2] LEONARDO MAGGI,[2] WENDY C. MARTIN,[1] VALERIA PRADO,[2] JAMES B. KAPER,[1] AND MYRON M. LEVINE[1]*

Center for Vaccine Development, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201,[1] and Unidad de Microbiologia, Facultad de Medicina, Universidad de Chile, Sede Oriente, Santiago, Chile[2]

Received 3 May 1990/Accepted 8 August 1990

A simple and economical method was developed for using biotinylated DNA probes to hybridize with bacterial colonies belonging to the various categories of diarrhea-causing *Escherichia coli*. Simplification and cost containment were achieved by using Whatman no. 541 filter papers instead of nitrocellulose, by minimizing the concentration of proteinase K (an expensive but necessary reagent used to pretreat the colony blots prior to hybridization with biotin-labeled DNA probes) and by reusing hybridization solution containing labeled probe DNA. After exposing the colony blots to lysing solution and steam, followed by lysozyme (1.5 mg/ml), sucrose (25%), and proteinase K (10 μg/ml) treatments, biotinylated probes were used to detect enterotoxigenic, enteropathogenic, enterohemorrhagic, diffuse adherence, and enteroinvasive categories of diarrhea-causing *E. coli* with a high level of sensitivity and specificity. Three independent observers who were experienced in reading DNA blots recorded remarkably similar results, while less satisfactory results were obtained when the blots were read by an inexperienced observer. This technique will be useful in laboratories in which radioactive isotopes are unavailable or impractical and in which budgets are restricted.

Prior to the availability of DNA probes, the epidemiology of diarrhea-causing *Escherichia coli* was studied by using immunoassays and bioassays to phenotypically identify virulence factors, such as toxins. This was cumbersome and expensive. The development of DNA probes that detect isolates belonging to the major categories of diarrhea-causing *E. coli* with a high degree of sensitivity and specificity was an important advance, enabling epidemiological studies to be supported by a single assay (2, 6, 14, 19, 25, 26). However, perhaps with the exception of enterohemorrhagic *E. coli* (EHEC) (13, 15, 17), the other categories of diarrhea-causing *E. coli*, including enterotoxigenic *E. coli* (ETEC) (2, 6, 13, 14, 26), enteroinvasive *E. coli* (EIEC) (2, 6, 13, 14, 25, 26), enteropathogenic *E. coli* (EPEC) (2, 6, 13, 14, 20, 26), diffuse adherence *E. coli* (DAEC) (14), and enteroaggregative *E. coli* (1–4), cause disease primarily in less-developed countries.

Early methods incorporated [α-^{32}P]dATP into the DNA probes as a marker and used nitrocellulose or Whatman no. 541 paper filters as a solid support (8, 15, 16, 19, 20, 22, 24, 27). [α-^{32}P]dATP can now be replaced with biotin-dATP (7, 9, 10, 11, 21). Using Whatman no. 541 paper filters and biotin-dATP would make it feasible to use this technique for large-scale epidemiological studies in laboratories with limited budgets. The biotinylated probe method exploits the high affinity of streptavidin for biotin-labeled molecules in a sandwich system analogous to an indirect enzyme-linked immunosorbent assay. A streptavidin-alkaline phosphatase conjugate is used to colorimetrically detect biotin-labeled DNA probes which have hybridized to target DNA present on the filter paper.

* Corresponding author.

Herein we describe a simple, economical method for using biotin-labeled DNA probes that is amenable to screening large numbers of *E. coli* colony blots. The technique was designed for an epidemiological field study of *E. coli* diarrhea in two cohorts of children under long-term surveillance in Santiago, Chile.

MATERIALS AND METHODS

***E. coli* strains.** A series of well-characterized ETEC, EPEC, EHEC, EIEC, and DAEC strains from the Center for Vaccine Development collection were used in this study.

Colony blots. The *E. coli* strains were inoculated onto MacConkey or Luria agar plates (40 colonies per plate, plus a positive and negative control) in a grid pattern. After overnight growth at 37°C, a Whatman no. 541 filter paper was pressed evenly over the surface of the plate and removed, lifting the colonies with the filter paper. Filters were placed with the colony side up in a petri dish containing a Whatman no. 3 filter paper saturated with a 0.5 M NaOH–1.5 M NaCl solution and steamed in an autoclave (with the door closed but not sealed) for 10 min to lyse bacteria and denature DNA (16). The Whatman no. 541 filters were placed upon another Whatman no. 3 filter paper saturated with 1.0 M Tris hydrochloride, pH 7.0, and 2.0 M NaCl for 10 min to neutralize the NaOH. The filters were air dried and either used immediately or saved.

Treatment of filters to remove colony debris. The effectiveness of lysozyme-sucrose and proteinase K in removing bacterial debris was assessed by exposing colony blots to lysozyme-sucrose alone, proteinase K alone, a combination of the two, or neither. A modification of the method of Haas and Fleming was used (5).

For treatment with lysozyme-sucrose alone, filters were rinsed three times in cold (4°C) 0.05 M Tris hydrochloride,

TABLE 1. Effect of lysozyme-sucrose and proteinase K on hybridization

Treatment[a]	Observer[b]	Result with DNA probe[c]: EAF		LT		EIEC		EHEC		DAEC	
		Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
None	1	UR	UR	90	100	UR	UR	100	100	UR	UR
	2	UR	UR	100	100	UR	UR	100	100	UR	UR
	3	UR	UR	100	100	UR	UR	100	100	UR	UR
	4	UR	UR	90	100	UR	UR	90	100	UR	UR
LS	1	100	100	100	100	95	50	100	100	100	80
	2	100	100	100	100	80	85	100	100	95	95
	3	100	100	100	100	85	100	100	100	100	90
	4	100	100	100	100	85	60	100	100	100	90
PK	1	95	95	100	100	75	55	100	100	90	95
	2	95	100	100	100	50	65	100	100	80	100
	3	95	100	100	100	35	80	100	100	80	100
	4	95	100	100	100	70	35	100	100	UR	UR
LS + PK	1	100	100	100	100	85	90	100	100	85	90
	2	100	100	100	100	85	95	100	100	85	95
	3	100	100	100	100	85	100	100	100	85	90
	4	100	75	100	100	85	85	100	100	85	85

[a] LS, Lysozyme (1.5 mg/ml) and 25% sucrose; PK, proteinase K (100 μg/ml).
[b] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not colony blots.
[c] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives); Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives); UR, unreadable.

pH 8.0; placed in 1.5 mg of lysozyme (L 6876; Sigma Chemical Co., St. Louis, Mo.)–25% sucrose per ml of cold (4°C) 0.05 M Tris hydrochloride (pH 8.0) (2 to 3 ml per filter) for 10 min; and then rinsed with vigorous agitation in prewarmed (42°C) SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) (9) (step 1).

For treatment with proteinase K alone, filters were rinsed in prewarmed (42°C) SSC and incubated in 100 μg of proteinase K (P-0390; Sigma) per ml of SSC (2 to 3 ml per filter) for 1 h at 42°C (9) (step 2).

These two steps were combined for filters treated with both lysozyme-sucrose and proteinase K. All filters were subsequently rinsed with SSC at ambient temperature and air dried (step 3).

Evaluation of different concentrations of proteinase K. Filters were treated as described in steps 1 and 2. The proteinase K concentrations used included 10, 100, and 1,000 μg/ml. To assess the reproducibility of the technique, additional experiments using 10 or 100 μg of proteinase K per ml were performed by using three of the five probes.

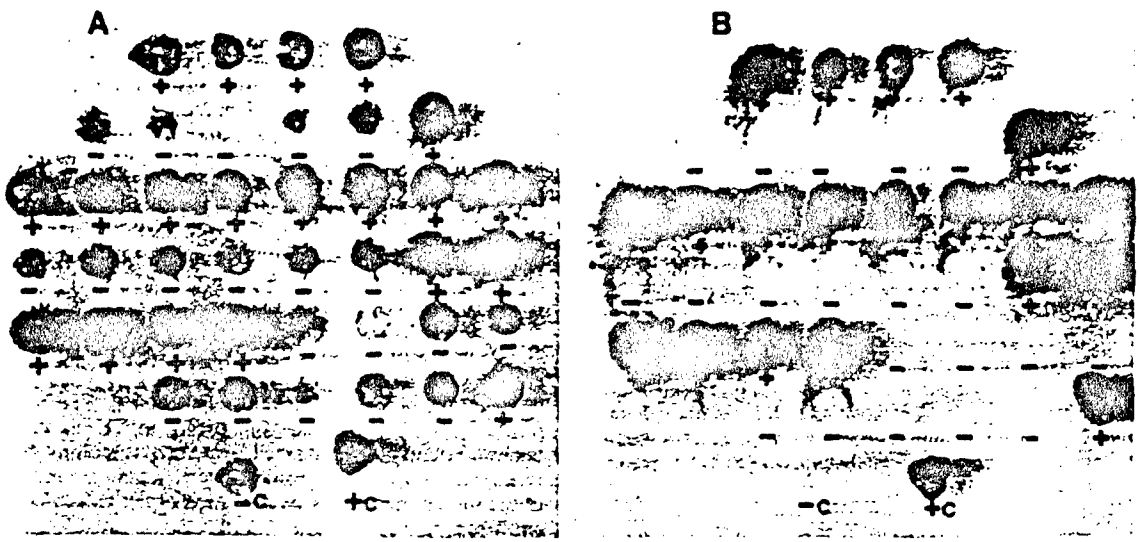

FIG. 1. Whatman filter paper containing EHEC and other *E. coli* colonies hybridized with the EHEC DNA probe. (A) Filter not pretreated with lysozyme-sucrose or proteinase K; (B) filter pretreated with lysozyme-sucrose followed by proteinase K.

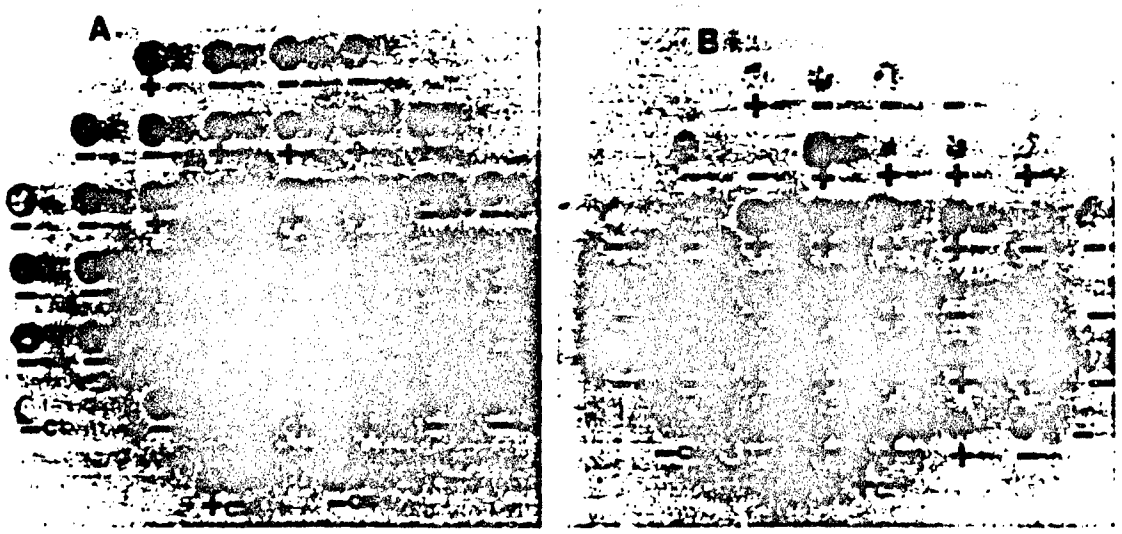

FIG. 2. Whatman filter paper containing EIEC and other *E. coli* colonies hybridized with the EIEC DNA probe. (A) Filter not pretreated with lysozyme-sucrose or proteinase K. (B) filter pretreated with lysozyme-sucrose followed by proteinase K.

TABLE 2. Effect of proteinase K concentration on hybridization

Treatment[a]	Observer[b]	Result with DNA probe[c]									
		EAF		LT		EIEC		EHEC		DAEC	
		Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
LS + 10 μg of proteinase K per ml											
Experiment 1	1	100	95	100	100	90	90	100	95	85	100
	2	100	95	100	100	90	85	100	95	85	100
	3	100	100	100	100	80	100	100	95	85	100
	4	100	95	100	100	95	85	100	95	90	100
Experiment 2	1	95	100	100	100	60	95	ND	ND	ND	ND
	2	95	95	100	100	70	95	ND	ND	ND	ND
	3	100	100	100	100	75	80	ND	ND	ND	ND
	4	100	50	100	35	75	80	ND	ND	ND	ND
LS + 100 μg of proteinase K per ml											
Experiment 1	1	100	90	100	100	85	95	100	100	85	100
	2	100	95	100	100	85	90	100	100	85	100
	3	100	100	100	100	80	100	100	100	85	100
	4	100	95	100	100	85	95	100	100	85	95
Experiment 2	1	80	100	100	100	70	95	ND	ND	ND	ND
	2	90	95	100	100	70	90	ND	ND	ND	ND
	3	95	100	100	100	70	90	ND	ND	ND	ND
	4	100	85	100	45	75	45	ND	ND	ND	ND
LS + 1 mg of proteinase K per ml	1	100	90	100	100	95	80	100	95	100	90
	2	100	100	50	100	90	85	100	100	100	90
	3	100	100	100	100	80	100	100	100	100	90
	4	100	95	95	100	85	90	100	100	100	90

[a] LS, Lysozyme (1.5 mg/ml) and 25% sucrose.
[b] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not DNA colony blots.
[c] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives). Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives). ND, not done.

TABLE 3. Reuse of hybridization solution

Times used	Observer[a]	Result with DNA probe[b]			
		EAF		EHEC	
		Sens	Spec	Sens	Spec
1	1	100	100	100	100
	2	100	100	100	100
	3	100	100	100	100
	4	100	100	100	100
2	1	100	100	100	100
	2	100	95	100	100
	3	100	95	100	100
	4	100	80	100	100
3	1	100	100	100	100
	2	100	100	100	100
	3	100	100	100	100
	4	100	90	100	100
4	1	100	100	100	95
	2	100	100	100	100
	3	100	100	100	100
	4	100	95	100	95

[a] Observers 1, 2, and 3 were experienced with reading DNA colony blots, and observer 4 was experienced with reading immunoblots, but not DNA colony blots.

[b] Sens, Sensitivity; defined as the number of true positives correctly identified/20 (total number of true positives); Spec, specificity; defined as the number of true negatives correctly identified/20 (total number of true negatives).

Preparation of biotinylated probes. DNA probes to detect EHEC, EPEC, EIEC, heat-labile enterotoxin (LT)-producing ETEC, and DAEC were prepared. The LT probe is a 1-kilobase *Bam*HI fragment derived from pWD299 (12, 19, 23). Briefly, the *Hin*cII fragment was removed from pWD299. *Bam*HI linkers were attached, and this fragment was cloned into pACYC184 and called pCVD403. The EHEC DNA probe is a 3.4-kilobase *Hin*dIII fragment from pCVD419 (15). The EPEC adherence factor (EAF) probe for detecting EPEC is a 1-kilobase *Sal*I-*Bam*HI fragment derived from strain E2348/69 (20). The EIEC probe is a 2.5-kilobase *Hin*dIII fragment of pSF55 derived from the epithelial cell invasiveness plasmid (pInv) of the EIEC strain *E. coli* 11 (22, 27). The DAEC probe is a 390-base-pair *Pst*I fragment from pSLM862 cloned into pUC8 (5).

The DNA probes were labeled with biotin-7-dATP (Bethesda Research Laboratories, Gaithersburg, Md.) by nick translation (18). Separation of unincorporated biotin-7-dATP from labeled probe DNA was not necessary. Immediately prior to use, probes were denatured to single strands of DNA by boiling for 10 min and then chilled on ice to prevent renaturation.

Hybridization. The processed filters were hybridized overnight at 42°C by a modification of the method of Sethabutr et al. (21) in a hybridization solution containing 45% formamide, 4× Denhardt solution (1× Denhart solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 4× SET buffer (1× SET buffer is 0.15 M NaCl, 0.03 M Tris hydrochloride [pH 8.0], and 1 mM EDTA), 0.5% sodium dodecyl sulfate (SDS), 24 μg of heat-denatured salmon sperm DNA per ml, and 40 ng of probe DNA per ml (2 ml of hybridization solution per filter).

After overnight hybridization, the filters were washed twice for 3 min in room temperature 2× SSC–0.1% SDS, twice for 3 min in room temperature 0.2× SSC–0.1% SDS, and twice at 50°C for 15 min in 0.16× SSC–0.1% SDS (BluGENE Nonradioactive Nucleic Acid Detection System; Bethesda Research Laboratories). Filters were then rinsed three times in 250 ml of 2× SSC to remove the SDS and visualized immediately.

Visualization of hybridization reaction. Colony blots were blocked for 1 h at 62°C in 3% bovine serum albumin (Sigma) dissolved in buffer 1 (0.1 M Tris hydrochloride (pH 7.5) and 0.15 M NaCl) prepared according to instructions accompanying the BluGENE detection system. It is important to keep the temperature of the blocking solution below 65°C, or it will gel and the filters will not be usable. Filters were placed directly into a solution of streptavidin-alkaline phosphatase (Bethesda Research Laboratories) at a concentration of 1 μg/ml in buffer 1 for 10 min at room temperature. Excess streptavidin-alkaline phosphatase was removed from the colony blots by washing them twice in buffer 1, followed by equilibration in buffer 2 (0.2 M Tris hydrochloride, pH 9.5; 0.1 M NaCl; and 50 mM $MgCl_2$). Positive colonies were identified by placing filters in a developing solution consisting of 1.5 μg of Nitro Blue Tetrazolium (Bethesda Research Laboratories) and 0.6 μg of 5-bromo-4-chloro-3-indolylphosphate (BRL) per ml of buffer 2. The signal from positive colonies was easily distinguishable from background in 1 to 3 h.

Blinded observers. All processed filters were coded and given to four independent observers to be read, including three observers experienced in reading biotinylated DNA probe blots and one experienced in reading immunoassay colony blots but not DNA blots.

Reuse of hybridization solution. Colony blots containing 20 EAF or EHEC out of 40 colonies were prepared as described above. The filters were identically treated with lysozyme-sucrose followed by proteinase K (100 μg/ml) as described above in steps 1 and 2. The filters were hybridized one filter at a time by using the same hybridization solution containing either the EAF or EHEC probe at 40 ng/ml in a volume of 10 ml per filter. Filters were washed and visualized as described above. Solutions containing DNA probes were stored at 4°C between hybridizations.

RESULTS

Lysozyme-sucrose versus proteinase K. The effects of pretreating filters with lysozyme-sucrose and proteinase K as a preliminary step to hybridization with the EAF, LT, EIEC, EHEC, or DAEC biotin-labeled DNA probe are shown in Table 1. Results varied markedly, depending on the particular probe. With two probes (LT and EHEC), high levels of sensitivity and specificity were obtained even without enzyme treatment (Fig. 1). However, the remaining three probes required that the filters be pretreated with at least one enzyme to obtain satisfactory results. Filters hybridized with the EIEC probe were not easily read unless they were pretreated with both lysozyme-sucrose and proteinase K. This is illustrated in Fig. 2, which represents blots that were either not pretreated (Fig. 2A) or treated with both lysozyme-sucrose and proteinase K (Fig. 2B) prior to hybridization with the EIEC probe.

Optimal proteinase K concentration. Experiments were performed to determine the lowest concentration of proteinase K that would give satisfactory sensitivity and specificity with all five DNA probes (Table 2). Following initial exposure of the filters to lysozyme-sucrose, treatment with the lowest concentration of proteinase K (10 μg/ml) gave as satisfactory results overall as treatment with higher concentrations did (Table 2).

Additional experiments were carried out to verify the reproducibility of the assay when low concentrations of proteinase K were used. After initial treatment with lysozyme-sucrose, filters were exposed to 10 or 100 μg of proteinase K per ml prior to hybridization with the LT, EAF, or EIEC DNA probe. Results are shown in Table 2 (experiment 2). As before, the experienced observers recorded satisfactory sensitivity and specificity with filters treated with lysozyme-sucrose followed by proteinase K in the lowest concentration of 10 μg/ml. The EIEC probe again gave the least satisfactory results.

Reuse of hybridization solution. A final set of experiments was undertaken to show that the hybridization solutions containing biotinylated probes can be reused (Table 3). EAF and EHEC probes were used in these experiments. At an initial probe concentration of 40 ng/ml, colony blots containing 20 EHEC colonies were sequentially hybridized three additional times with the EHEC probe, with identical results (100% sensitivity and specificity recorded by all four observers). Similar results were obtained with the EPEC probe in four sequential hybridizations of colony blot filters containing 20 EPEC strains.

DISCUSSION

Previous attempts to use biotinylated DNA probes for colony blot hybridization have given mixed results (7, 9, 10, 11, 21). We set out to develop a simple, practical, low-cost method for using biotinylated DNA probes to hybridize with bacterial colony blots, so that laboratories of moderate sophistication in less-developed countries can support epidemiologic studies. Substituting Whatman no. 541 filter papers for nitrocellulose, as originally suggested by Sethabutr et al. (21), markedly lowers costs ($0.10 for one Whatman no. 541 filter versus $1.92 for one nitrocellulose filter). Paper filters also simplify the procedure, since they are much easier to handle than nitrocellulose and do not require baking.

One potential problem with biotinylated DNA probes in screening colony blots is interpretation of the hybridization results in the face of nonspecific background color development. Treatment of the colony blots with enzymes can remove bacterial debris and improve the quality of the hybridizations. Previous published protocols used proteinase K in concentrations from 200 μg/ml to 1 mg/ml (9, 21). However, since proteinase K is quite costly, we sought to verify its usefulness. While such treatment was not required for two probes (LT and EHEC), for the remaining DNA probes tested, the use of both lysozyme-sucrose and a low concentration of proteinase K (10 μg/ml) was indeed necessary to achieve acceptable sensitivity and specificity (Tables 1 and 2).

Biotinylated DNA probes can be stored for long periods of time without the instability that occurs with radioactive probes, thereby making it theoretically possible to reuse hybridization solutions containing biotin-labeled probes (Table 3). Such reuse diminishes technician time, conserves reagents and DNA probe fragments, and simplifies the procedure by eliminating the need to prepare fresh hybridization solutions for each experiment.

An important practical lesson learned from these studies is that, not surprisingly, the prior experience of the observer in reading DNA hybridizations markedly influences the sensitivity and specificity achieved in the assay. There was extraordinary correlation among the results recorded by the three independent observers experienced in reading filters hybridized with DNA probes. In contrast, the erratic results of the observer experienced in reading immunoblots but not DNA blots underscore the need to include a period of training for any observer chosen to interpret the processed filters. We have successfully trained several visiting scientists to become proficient in interpreting results of the biotinylated probe method with 3 to 4 weeks of intensive instruction. This includes comparing results of two replicate sets of filters, one hybridized with a ^{32}P-labeled probe and the other with a biotinylated probe, under the guidance of an experienced observer.

The colony blot hybridization protocol described here is economical, gives reproducible and relatively easy-to-interpret results, and allows one to capitalize upon the inherent advantages of biotinylated DNA probes, such as their ease and safety in handling and their long shelf life. Laboratories with limited budgets and those that do not have the facilities to use radioisotopes will benefit from this technique.

ACKNOWLEDGMENTS

This work was supported by research contract DAMD17-87C-7022 from the United States Army Medical Research and Development Comand.

We thank Bernadette Baudry and David Maneval for their help with reading colony blots.

LITERATURE CITED

1. **Baudry, B., S. Savarino, P. Vial, J. B. Kaper, and M. M. Levine.** 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J. Infect. Dis. 161:1249–1251.
2. **Bhan, M. K., N. Bhandari, S. Sazawal, J. Clemens, P. Raj, M. M. Levine, and J. B. Kaper.** 1989. Descriptive epidemiology of persistent diarrhoea among young children in rural northern India. Bull. W.H.O. 67:281–288.
3. **Bhan, M. K., V. Khoshoo, H. Sommerfelt, P. Raj, S. Sazawal, and R. Srivastava.** 1989. Enteroaggregative *Escherichia coli* and *Salmonella* associated with nondysenteric persistent diarrhea. Pediatr. Infect. Dis. J. 8:499–502.
4. **Bhan, M. K., P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal.** 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. J. Infect. Dis. 159:1061–1064.
5. **Bilge, S. S., C. R. Clausen, W. Lau, and S. L. Moseley.** 1989. Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2 cells. J. Bacteriol. 171:4281–4289.
6. **Black, R. E., G. L. De Romana, K. H. Brown, N. Bravo, O. G. Bazalar, and H. C. Kanashiro.** 1989. Incidence and etiology of infantile diarrhea and major routes of transmission in Huascar, Peru. Am. J. Epidemiol. 129:785–799.
7. **Chatkaeomorakot, A., P. Echeverria, D. N. Taylor, K. A. Bettelheim, N. R. Blacklow, O. Sethabutr, J. Seriwatana, and J. Kaper.** 1987. HeLa cell-adherent *Escherichia coli* in children with diarrhea in Thailand. J. Infect. Dis. 156:669–672.
8. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA 72:3961–3965.
9. **Haas, M. J., and D. J. Fleming.** 1986. Use of biotinylated DNA probes in colony hybridization. Nucleic Acids Res. 14:2376.
10. **Haas, M. J., and D. J. Fleming.** 1988. A simplified lysis method allowing the use of biotinylated probes in colony hybridization. Anal. Biochem. 168:239–246.
11. **Huovinen, S., P. Huovinen, and G. A. Jacoby.** 1988. Reliability of biotinylated DNA probes in colony hybridization: evaluation of an improved colony lysis method for detection of TEM-1 beta-lactamase. Mol. Cell. Probes 2:83–85.
12. **Lanata, C. F., J. B. Kaper, M. M. Baldini, R. E. Black, and M. M. Levine.** 1985. Sensitivity and specificity of DNA probes with the stool blot technique for detection of *Escherichia coli*

enterotoxins. J. Infect. Dis. 152:1087–1090.
13. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155:377–389.
14. **Levine, M. M., V. Prado, R. Robbins-Browne, H. Lior, J. B. Kaper, S. L. Moseley, K. Gicquelais, J. P. Nataro, P. Vial, and B. Tall.** 1988. Use of DNA probes and Hep-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. J. Infect. Dis. 158:224–228.
15. **Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachsmuth.** 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of 0157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. 156:175–182.
16. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid 10:296–298.
17. **MacDonald, K. L., M. J. O'Leary, M. L. Cohen, P. Norris, J. G. Wells, E. Noll, J. M. Kobayashi, and P. A. Blake.** 1988. *Escherichia coli* 0157:H7, an emerging gastrointestinal pathogen, results of a one-year, prospective, population-based study. J. Am. Med. Assoc. 259:3567–3570.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. p. 109–112. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Moseley, S. L., I. Hug, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. 142:892–898.
20. **Nataro, J. P., J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. 152:560–565.
21. **Sethabutr, O., S. Hanchalay, P. Echeverria, D. N. Taylor, and U. Leksomboon.** 1985. A non-radioactive DNA probe to identify *Shigella* and enteroinvasive *Escherichia coli* in stools of children with diarrhoea. Lancet ii:1095–1097.
22. **Small, P. L. C., and S. Falkow.** 1986. Development of a DNA probe for the virulence plasmid of *Shigella* spp. and enteroinvasive *Escherichia coli*. p. 121–124. *In* L. Leive (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
23. **So, M., W. S. Dallas, and S. Falkow.** 1978. Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. Infect. Immun. 21:405–411.
24. **Taub, F., and E. B. Thompson.** 1982. An improved method for preparing large arrays of bacterial colonies containing plasmids for hybridization: *in situ* purification and stable binding of DNA on paper. Anal. Biochem. 126:222–230.
25. **Taylor, D. N., P. Echeverria, T. Pal, O. Sethabutr, S. Saiborisuth, S. Sricharmorn, B. Rowe, and J. Cross.** 1986. The role of *Shigella* spp., enteroinvasive *Escherichia coli*, and other enteropathogens as causes of childhood dysentery in Thailand. J. Infect. Dis. 153:1132–1138.
26. **Taylor, D. N., P. Echeverria, C. Pitarangsi, J. Seriwatana, O. Sethabutr, L. Bodhidatta, C. Brown, J. E. Herrmann, and N. R. Blacklow.** 1988. Application of DNA hybridization techniques in the assessment of diarrheal disease among refugees in Thailand. Am. J. Epidemiol. 127:179–187.
27. **Wood, P. K., J. G. Morris, Jr., P. L. C. Small, O. Sethabutr, M. R. F. Toledo, L. L. Trabulsi, and J. B. Kaper.** 1986. Comparison of DNA probes and the Sereny test for identification of invasive *Shigella* and *Escherichia coli* strains. J. Clin. Microbiol. 24:498–500.

APPENDIX D

EPIDEMIOLOGIC STUDIES OF ESCHERICHIA COLI DIARRHEAL INFECTIONS IN A LOW SOCIOECONOMIC LEVEL PERIURBAN COMMUNITY IN SANTIAGO, CHILE

Myron M. Levine, M.D., D.T.P.H.[1], Catterine Ferreccio, M.D., M.P.H.[1,2], Valeria Prado, M.D.[3], Marisol Cayazzo, R.N., M.P.H.[2], Paulina Abrego, R.N.[2], Juan Martinez, B.S.[3], Leonardo Maggi, M.D.[3], Mary M. Baldini, Ph.D.[1], Nellie Contreras, M.D.[2], Wendy Martin, B.S.[1], David Maneval, B.S.[1], Bradford Kay, Dr.P.H.[1], Linda Guers, B.S.[1], Hermy Lior, M.S.[4], Steven S. Wasserman, Ph.D.[1], James P. Nataro, M.D., Ph.D.[1]

Center for Vaccine Development[1]
Division of Geographic Medicine, Department of Medicine,
University of Maryland School of Medicine
Baltimore, Maryland 21201

Program for the Control of Typhoid Fever and Enteric Diseases,
Ministry of Health, Chile[2]

Microbiology Unit, Faculty of Medicine, University of Chile,
Sede Oriente, Santiago, Chile[3]

Enteric Reference Laboratory[4],
Canadian Centre for Disease Control,
Ottawa, Ontario

ABSTRACT

Background. The incidence of diarrheal disease due to the six recognized categories of diarrheagenic *Escherichia coli* was determined in two cohorts of children in a low socioeconomic peri-urban community in Santiago, Chile where virtually the entire population has access to potable water.

Methods. Two cohorts of children were followed by means of twice weekly household visits to detect episodes of diarrheal disease. An age cross-sectional cohort was assembled by enrolling 340 children (85 < 12 months, 85 12-23 months, 85 24-35 months and 85 36-47 months of age). A newborn cohort was assembled by enrolling 10-12 newborns each month for 12 consecutive months. When an episode of diarrhea occurred, stool cultures were obtained on two consecutive days and *E. coli* colonies were hybridized with non-radioactive DNA probes specific for enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enteroaggregative *E. coli* and diffuse adherence *E. coli*. For each child with diarrhea, *E. coli* colonies from stool cultures of a matched control without diarrhea within the cohort were also tested.

Results. In both cohorts, enterotoxigenic were important pathogens. Expression of fimbrial colonization factor antigens was markedly more common in isolates having both heat-labile enterotoxin and heat-stable enterotoxins or heat-stable enterotoxin alone compared with strains elaborating only heat-labile enterotoxin. The age-related pathogenicity of enteropathogenic *E. coli* was particularly evident in the newborn cohort where during the first year of life (0-11 month olds), EPEC were found significantly more often in cases of diarrhea (21 isolations/314 episodes of diarrhea cultured) than in age-matched controls (8 isolations/349 cultures) ($p = 0.011$); beyond this age rates of isolation were similar between cases and controls. In contrast, the relative risk of isolation of diffuse adherence *E. coli* increased with age in the age cross-sectional cohort; the difference in rate of isolation between cases and controls was significant ($p = 0.0024$). Enteroinvasive *E. coli* and enterohemorrhagic *E. coli* were infrequently found. Isolates of the latter possessed the same virulence properties typically found in strains from patients with the hemolytic-uremic syndrome or hemorrhagic colitis. Enteroaggregative *E. coli* were encountered with equal frequency between cases and controls in each cohort.

Conclusions. Overall, children in this low socioeconomic community suffered a relatively low incidence of diarrhea (2.1 episodes per infant per year). Nevertheless, using DNA probes for detection, a putative *E. coli* diarrheal pathogen was found in a large proportion of the diarrheal episodes, particularly in summer. Santa Julia is an attractive site to undertake field trials of efficacy of vaccines or other interventions against enterotoxigenic *E. coli* or other categories of diarrheagenic *E. coli*.

INTRODUCTION

Diarrheal diseases constitute a major cause of morbidity among infants and young children in the less-developed world (1). Identification of the etiologic agents associated with diarrheal disease in well-defined pediatric populations allows the relative importance of the pathogens to be assessed in different settings and can lead to prioritization of specific interventions and of allocation of resources for research (2-4). While it has been known for half a century that some strains of Escherichia coli can cause infant diarrhea (5,6), only during the past 15 years has it become apparent that diarrheagenic E. coli comprise a heterogeneous array of enteric pathogens which together can account for a considerable proportion of diarrheal disease (7).

Currently, five distinct categories of Escherichia coli are recognized that are associated with diarrheal disease, including enterotoxigenic E. coli (ETEC) (7), enteroinvasive E. coli (EIEC) (7), enteropathogenic E. coli (EPEC) (7), enterohemorrhagic E. coli (EHEC) (7) and enteroaggregative E. coli (EAggEC) E. coli (8-10); a sixth category, so-called diffuse adherence E. coli (DAEC) (11-13), may also include diarrheal pathogens but fewer incriminating data are available. Each of the five main categories of diarrheagenic E. coli possesses specific virulence properties such as characteristic interactions with intestinal epithelial cells (e.g., mechanisms of adherence, microvillus effacement or cell invasion) or elaboration of enterotoxins; many of these properties require the presence of specific virulence plasmids or phages (7). Isolates within each category tend to fall within characteristic O:H serotypes that differ by category (7).

ETEC

ETEC adhere to enterocytes by means of fimbrial colonization factors and elaborate heat-labile (LT) or heat-stable (ST) enterotoxins which result in watery diarrhea that can dehydrate infants. LT closely resembles cholera toxin in structure and action, while ST is a small peptide (18 or 19 amino acids). The major colonization factor antigens (CFAs) of ETEC include CFA/I and coli surface antigens (CS) 1-6. CS1, CS2 and CS3 represent a family of antigens that comprise CFA/II, while CS4, CS5 and CS6 comprise the CFA/IV family of antigens. All CFA/II strains express CS3 either alone or in

conjunction with CS1 or CS2. Similarly, all CFA/IV strains express CS6, either alone or in conjunction with CS4 or CS5.

EIEC

EIEC closely resemble Shigella and likewise have the capacity to invade enterocytes and multiply therein (14,15). EIEC also elaborate an enterotoxin that causes secretion of water and electrolytes by the small intestine (16); some individuals infected with EIEC manifest dysentery characterized by passage of scanty stools containing blood and mucus (17,18).

EPEC

EPEC have a chromosomal gene that encodes a 94 kD protein involved with intimate attachment accompanied by effacement of microvilli (19); expression of the intimate attachment and perhaps the microvillus-effacing factor is regulated by genes located on a plasmid (so-called EPEC Adherence Factor or EAF plasmid) (20,21) that also encodes a bundle-forming pilus (i.e. fimbrial) attachment factor (22). These virulence factors are present in EPEC of the major "classical" O:H serotypes that decades ago were incriminated as agents of diarrhea. In young infants, EPEC cause fever and mucoid watery diarrhea that can lead to dehydration ( ) and is sometimes accompanied by "toxemia" (23).

EHEC

Three virulence properties are recognized in EHEC: a plasmid-associated, fimbriae-mediated attachment to epithelial cells (24), phage-encoded expression of potent Shiga-like cytotoxins (25) and a capacity to induce intimate attachment (26) (encoded by a gene sharing considerable homology with the EPEC microvillus effacement factor) (27). Severe EHEC infection manifests clinically as afebrile hemorrhagic colitis (28) with some individuals going on to develop hemolytic-uremic syndrome ( ); in milder cases EHEC causes watery diarrhea.

EAggEC

EAggEC exhibit an aggregative pattern of adherence to HEp-2 cells in tissue culture and to human intestinal mucosa (10,30-33). This unique attachment requires the presence of a virulence

plasmid (30,34) that also encodes a novel EAggEC heat-stable enterotoxin (EAST) (35). EAggEC are associated with diarrhea, particularly persistent diarrhea (lasting > 14 days), in children in less-developed countries (8,9,13,36).

DAEC

DAEC are characterized by their fimbriae-mediated (11) pattern of adherence to HEp-2 cells (10,31); the fimbrial subunit gene is located on the chromosome (11). Enterotoxin production by DAEC has not yet been described. Studies to investigate DAEC as agents of diarrhea have provided conflicting data. DAEC have not caused diarrhea when fed to volunteers (37) and heretofore all field studies (8,9,13,38) save one (12) have isolated DAEC equally from stool cultures of diarrhea cases and matched controls.

Detection of Diarrheagenic E. coli

It is logistically cumbersome and expensive to identify diarrheagenic E. coli by phenotypically detecting virulence properties. Consequently, few comprehensive population-based surveys have been undertaken to study the relative importance and the epidemiologic characteristics of the different categories of diarrheagenic E. coli. Heretofore, epidemiologic studies have been limited to investigating only one or a few of the categories of diarrheagenic E. coli (39-45) and have generally involved relatively small numbers of subjects. The development of non-radioactive DNA probes that detect the distinct categories of diarrheagenic E. coli with high sensitivity and specificity (46,47) has paved the way for comprehensive, practical and economical epidemiologic studies. Herein we describe such a study carried out in a low socioeconomic level pediatric population in Santiago, Chile.

MATERIALS AND METHODS

Study site

The periurban low socioeconomic community of Santa Julia in Area Oriente, Santiago has been previously described in detail (48). The 133,909 inhabitants (of whom 31 percent are below 15 years of age) live in ramshackle housing within an area of 12.3 km^2. At the initiation of the study, a total of 15,525 children less than five years of age resided in Santa Julia, including 2,844 less than 12 months,

2,811 from 12 to 23 months, and 9,870 from 2 to 5 years of age. The Chilean National Health Service provides health care to 96 percent of the population of Santa Julia through a neighborhood health center, the Consultorio Santa Julia. Out-migration is approximately 8 percent annually.

Virtually all households in Santa Julia have access to potable water (68 percent have an inside tap); 64 percent of households have an inside toilet, 34 percent have access to a toilet outside the household and 3 percent are served by latrines (48). Less than one-half of households have a refrigerator.

Study participants

Household surveillance cohorts

Two separate cohorts of children were prospectively followed with active household surveillance, an age cross-sectional cohort and a birth cohort. Participation was restricted to pediatric subjects without chronic disease or serious congenital malformations and was accompanied by informed parental consent. The protocol was approved by the Ministry of Health of Chile and by ethics committees at the University of Maryland School of Medicine, the World Health Organization and the U.S. Department of Defense.

Age cross-sectional cohort. Participants in this cohort were a stratified sample of 360 Santa Julia children under five years of age, 90 falling within each one of four age strata: less than 12 months of age, 12-23 months, 24-35 months and 36-47 months of age. The sampling procedure to assemble this cohort has been previously described in detail (48). The cohort was assembled in November, 1986 and surveillance for diarrhea due to E. coli began one month later (December, 1986). When children reached 60 months of age they were dropped from the study and replaced with children less than 12 months of age.

Birth cohort. Beginning in March, 1987, approximately 12 newborn infants each month for a period of 12 consecutive months were enrolled in a birth cohort and prospectively followed until 24 months of age. The structure of this cohort, in contrast to the age cross-sectional cohort, assured that ample surveillance data and laboratory specimens were available from young infants.

Surveillance for diarrhea in the cohorts

The household of each participating child in the two cohorts was visited twice weekly by a trained public health nurse or nurse auxiliary who queried the caretaker about the occurrence of diarrheal illness (48). Systematic questioning focused on the consistency, number, and character (i.e., watery, loose, bloody) of stools that occurred during each 24-hour period since the previous visit: responses were recorded using a precoded questionnaire. Accompanying symptoms (e.g., vomiting and lethargy) as well as objective signs of dehydration were noted and the rectal temperature was recorded.

Oral glucose-electrolytes solution was provided by the nurse when appropriate. Criteria for a child to be seen by the study pediatrician included signs of dehydration, high fever, marked lethargy, or overt dysentery (blood in stools). Children with dysentery or with persistent diarrhea (> 14 days) received oral trimethoprim/sulfamethoxazole (49).

Surveillance for cases of diarrheal illness in the consultorio and in the hospital

From Monday to Friday (the days when the consultorio was open), a health auxiliary recorded the visits of every child less than sixty months of age who visited Consultorio Santa Julia with a chief complaint of diarrheal illness. The clinician caring for the child recorded the number and character of stools, the presence of fever, vomiting, signs of dehydration and whether antibiotics were used.

The Calvo MacKenna Children's Hospital serves all of Area Oriente, including Santa Julia. Hospital surveillance was maintained by sending a nurse on Monday through Friday to review all hospital admissions for diarrheal illness in the emergency room, infant/toddler and Infectious Disease Unit services. A single stool culture (see below) was obtained from every hospitalized child coming from the community of Santa Julia and clinical data were systematically collected until discharge.

Clinical definitions

Diarrhea is defined as an overt change in the child's stool pattern, characterized by an increase in the frequency (to at least three stools per 24-hour period) and a decrease in the consistency of stools to an unformed state. *Dysentery* refers to loose stools that contain gross blood, with or without

mucus.

An episode of diarrhea is considered to have commenced after 7 consecutive days without diarrhea and to have ended on the first day that was followed by 7 consecutive days without diarrhea. *Episodes of diarrhea associated with a particular category of E. coli* are defined as diarrhea (see above) accompanied by the isolation of diarrheagenic E. coli from coprocultures taken at the time of illness. *Asymptomatic infection due to the various categories of diarrheagenic E. coli* refers to isolation of such E. coli from a child in the absence of diarrhea.

Selection of control children

After the cohorts were assembled, each child was matched with another child of the same age (within six months) and sex as a "one-way" control, as previously described (48).

Clinical specimens

When an episode of diarrheal illness was detected, stool specimens or rectal swabs were obtained for bacteriologic culture on two consecutive days from the ill child; analogous specimens for culture were obtained on the same two consecutive days from a predetermined age-matched asymptomatic control child in the cohort. A sample of stool on a cotton-tipped applicator stick, or a rectal swab obtained by the nurse, was inoculated into a screw-top tube containing Cary Blair transport medium and kept in a styrofoam cooler with polyethylene glycol "cold packs" until arrival at the laboratory. In general the samples reached the laboratory within several hours.

Laboratory methods

Stool specimens were inoculated onto MacConkey's agar and incubated 18-24 h at 37°C. Individual colonies (three per specimen) were sub-cultured onto trypticase soy agar and colonies were then transferred to Whatman filter paper for subsequent hybridization with DNA probes; the colonies were also inoculated into Dorset egg slants and preserved for further testing at a later point, such as serotyping and detecting colonization factor antigens. Microbiologic aspects of the various DNA probes employed have been described previously (13,46,50,51-54). Lactose-positive coliform colonies were routinely examined with DNA probes to detect ETEC, EIEC, EPEC, EHEC, EAggEC and DAEC. ETEC

were identified using probes for heat-labile (LT) and heat-stable enterotoxins (ST) (13,46,50); EIEC were identified using the probe for the 140 MD invasiveness plasmid (51); EPEC were detected using a probe for the EPEC Adherence Factor (EAF) plasmid (54); EHEC were screened for using the probe for the 60 mD EHEC virulence plasmid (52) and positives were subsequently tested using probes for Shiga-like Toxin 1 and 2 (55); DAEC were identified using a probe for the gene encoding the subunit of the fimbria that mediates diffuse adherence; EAggEC were detected using a probe for the EAggEC plasmid (that encodes aggregative adherence and EAST). The ST probe was from a commercial kit and consisted of a synthetic oligonucleotide linked to alkaline phosphatase (47). All the other probes consisted of specific cloned fragments of DNA that were purified, made single stranded and biotin-labelled by the investigators for use as diagnostic reagents (46). Each filter to be hybridized with a specific probe was spotted with 40 test colonies of E. coli and an appropriate positive and negative control colony (46). Lactose-negative colonies were not tested with the EIEC probe. Previous studies in Santiago showed that the vast majority of EIEC are lactose-positive (56).

Fimbrial colonization factor antigen I (CFA I), coli surface (CS) antigens 1-3 of the CFA/II family and CS4-6 of the CFA/IV family and PCF O159:H4 fimbriae were detected by colony blot immunoassay (57) using monospecific antisera prepared in rabbits. O serogroups and H flagella serotypes were identified at the Canadian Centre for Disease Control, Ottawa, by standard tube agglutination techniques (58).

Epidemiologic measures

Within each age group, the incidence rate of diarrheal episodes due to the various categories of E. coli per child per 12 months of observation was calculated by dividing the total number of episodes of that category that were detected by the total child months of observation for children within that age group and multiplying by 12.

Statistical methods

Rates were compared by Chi square or Fisher's exact test (two tails). Wilcoxon rank sum test was used to analyze continuous variables.

RESULTS

Active surveillance of the age cross-sectional cohort

A total of 1178 episodes of diarrhea were detected in the cohort during 30 months of follow-up between December 1, 1986 and May 31, 1989. In 1081 (92 percent) of these episodes, stool samples were tested for the presence of diarrheagenic E. coli; in 1047 of these 1081 episodes, stool samples from matched controls were also tested. Table 1 shows the identification of diarrheagenic E. coli by probe technique from cases of diarrhea and controls tested during this 30 month period of observation.

ETEC

ETEC infections were common in the age cross-sectional cohort, being associated with 12.3 percent of cases overall. The rate of isolation of ETEC from cases (133/1081, 12.3%) was significantly greater than from controls (74/1063, 7.0%) ($p = 0.000039$). ETEC diarrheal infections peaked in the warm months of the year (Table 1). In the summer months of December through February, a total of 65 episodes of ETEC diarrhea were recorded during 2974 summer months of observation (2.2 cases/100 mos), while in the cool winter months of June through August, only 8 episodes of ETEC diarrhea were observed during 2024 months of winter surveillance (0.4 cases/100 mos) ($p = 0.00001$). The incidence of ETEC diarrhea by age group in this cohort is shown in Table 2 along with the incidence rates of diarrhea associated with the other categories of diarrheagenic E. coli. The highest incidence of ETEC diarrheal illness occurred in the first year of life and diminished slightly but steadily thereafter with increasing age. These data suggest that during the first two years of life in Santa Julia a child has a 50% likelihood of experiencing an episode of ETEC diarrhea and by five years of age this becomes a 90% likelihood.

ETEC infections analyzed by toxin profile and by age are shown in Table 3. Of the 133 episodes of diarrhea associated with ETEC, 31 (23%) yielded LT/ST strains, 34 (26% [illegible]only strains and 66 (50%) LT-only strains; two cases yielded both LT-only and ST-only isolates (1.5%). The highest relative risk of isolation between cases and controls was seen with LT/ST strains (RR = [illegible] Table 3), followed by ST-only strains (RR =2.2); the relative risk for LT-only strains was only 1.4.

CFA typing was performed on isolates from the first 96 of the 133 episodes of ETEC diarrhea detected in this cohort. Three of these infections yielded more than one ETEC strain (one child had a combined O128:NM,CS2/CS3 plus O153:H45,CFA/I infection, the second child had infection with both an ST-only strain expressing CFA/IV plus an LT-only strain without CFAs and the third child had dual infection with LT/ST and ST-only strain, neither bearing CFAs), whereas only a single ETEC strain was identified in the remaining 93 infections. Among the episodes caused by single ETEC strains, a fimbrial CFA was expressed in 41 of the 93 (44%) overall. CFA expression was closely correlated with toxin type, being found in 19/19 (100%) of the LT/ST infections and 16/28 (57%) of the ST-only infections but in just 6/46 (13%) of the LT-only ETEC infections. In these 41 ETEC episodes where the implicated strains were found to express CFAs, the CFA/II family was most common (25/41, 61%), followed by CFA/I (11/41, 27%) and finally the CFA/IV family of antigens (5/41, 12%). Among the isolates expressing one or more antigens of the CFA/II family, the combinations of CS1 and CS3 or CS2 and CS3 were encountered with equal frequency (44% each), whereas only 12% expressed CS3 alone. In contrast, among the five episodes caused by isolates expressing CFA/IV antigens, four were due to strains bearing CS6 alone, while one infection was caused by a strain expressing CS5 and CS6; no infections in this cohort were due to strains expressing both CS4 and CS6.

ETEC isolates from a proportion of the first 94 episodes of ETEC diarrhea in this cohort were serotyped. The most common O:H types included O6:H16 (N=16), O6:NM (N=15) among the LT/ST isolates, and O128:NM (N=14) and O153:H45 or O153:H non-typable (N=7) among the ST-only strains.

EPEC

EPEC diarrheal illness is known to be largely confined to infants less than six months of age (23), an age group not well represented in this cohort. Overall, there was no significant difference in the rate of isolation of EPEC between cases and controls (Tables 1 & 4), as would be expected in this cohort containing mainly toddlers and preschool children. Nevertheless, the difference in the rate of isolation of EPEC between cases and controls in this cohort (20/162, 12.3% versus 10/159, 6.3%) in infants below 12 months of age approached significance ($p = 0.09$) (Table 4); this 0-11 month age

group also manifested the highest relative risk (RR = 2.0).

The incidence of EPEC-associated diarrhea is shown in Table 2. Isolations of EPEC were more common in summer months (Table 1).

EIEC and EHEC

EIEC and EHEC infections were relatively uncommon (Table 1). The isolation of EIEC and EHEC in relation to age is summarized in Table 4 and the incidence by age is shown in Table 2. Overall the rate of isolation of EHEC was low in all age groups and was similar in cases and controls (Table 4). Of the 16 EHEC strains isolated from cases of diarrhea using the EHEC virulence plasmid probe, 13 were available for testing with probes that detect SLT1 and SLT2; 10 of the 13 were positive (4 SLT1, 3 SLT2 and 3 positive for both). EIEC were isolated more often from cases than controls but the difference was not significant. However, an age-specific pattern was discerned (Table 4). Among infants and toddlers below 24 months of age, the rate of isolation of EIEC in cases (11/443, 2.5%) and controls (11/434, 2.5%) was identical (RR = 1.0). In contrast, among children 24-59 months of age, an age group in which EIEC infections are purported to be clinically important (58a), EIEC were found more often in cases (20/638, 3.1%) than in controls (9/629, 1.4%) ($p = 0.059$) (RR = 2.2).

DAEC

DAEC infections were common, being isolated from 16.6 percent of cases and from 11.9 percent of controls; this difference was statistically significant ($p = 0.0024$). DAEC exhibited the same seasonal pattern as ETEC, being notably more common in the warm season (Table 1). The pathogenicity of DAEC appeared to increase with age as suggested by the increasing relative risk with increasing age (Table 4). In the youngest age group (0-11 month olds), the relative risk for isolation of DAEC was 1.1 but rose steadily in subsequent age groups to reach a value of 2.1 in children $\geq$ 48 months of age.

EAggEC

EAggEC were not isolated with greater frequency from cases than from controls. One hundred and three of the 1081 episodes of diarrhea where *E. coli* were tested with probes were persistent

(9.5%). EAggEC were associated with 17 of these 103 episodes (16.5%); of the 96 matched controls that were tested, EAggEC were isolated from the stools of 24 (25%).

Newborn cohort

Table 2 summarizes the child months of observation of children in this cohort, by age group, and provides the number of episodes and the incidence of diarrhea due to each category of diarrheagenic E. coli.

ETEC

The epidemiology of ETEC diarrheal infections in the newborn cohort was found to resemble remarkably the pattern seen in the age cross-sectional cohort. ETEC were associated with 8.2 percent of all diarrheal episodes in the newborn cohort, an isolation rate significantly higher than found in controls (3.0%) ($p = 0.00001$) (Table 5). Beyond five months of age, the incidence of ETEC diarrhea was similar for all age groups. The relative frequency of the different enterotoxin patterns among isolates from cases in this cohort was similar to the cross-sectional cohort. LT-only strains were found in 41% of cases, ST-only strains in 37% and LT/ST strains in 11%; the remaining 11% of cases yielded both LT-only and ST-only strains.

EPEC

In this cohort followed from birth, it was possible to examine closely the epidemiology of EPEC diarrhea. Over the entire period of surveillance, which included follow-up of all children until at least 24 months of age, the isolation of EPEC from cases (31 isolations from 662 episodes cultured) was not significantly different from controls (20 isolations among 658 cultures) ($p = 0.$) (Table 5). However, in this cohort one can clearly discern the relationship between age and pathogenicity of EPEC. During the first year of life (0-11 month olds), EPEC were found significantly more often in cases of diarrhea (21 isolations/314 episodes of diarrhea cultured) than in the age-matched controls (8 isolations/349 cultures) ($p = 0.00$). In contrast, the rate of isolation between cases and controls in children above 11 months of age was quite similar (10 isolations among 348 diarrheal episodes cultured) versus 12 isolations among 340 controls cultured) ($p =$ NS).

EIEC, EHEC, DAEC and EAggEC

In the newborn cohort EIEC were uncommon and were recovered in a similar rate from cases and controls (Table 5). EHEC infections were also uncommon and were found slightly more often in controls than in cases. As in the age cross-sectional cohort, DAEC were common but in the newborn cohort they were recovered equally in stool cultures of cases and controls. Overall, EAggEC were isolated at the same rate from cases of diarrhea and matched controls. A total of 77 of the 662 episodes of diarrhea in this cohort were persistent and 12 of these (15.6%) were associated with EAggEC. However, EAggEC were also recovered from 10 of the 77 matched controls without diarrhea (13.0%). Thus, in this cohort, EAggEC was not significantly associated with persistent diarrhea.

Consultorio and hospital surveillance

ETEC

The isolation of ETEC from children with diarrhea cared for at the consultorio or the hospital is shown in Table 6. Several points are worth making. First, among these more severely ill children detected by passive surveillance, ETEC infections constituted approximately the same proportion of all diarrhea cases as was seen in the cohorts where cases were actively detected by household visits. This point is illustrated in Table 6 where bacteriologic methods have been standardized by comparing results of the first stool culture (of the two consecutive daily cultures) obtained from children in the age cross-sectional cohort with results of the single stool culture obtained from children with diarrhea seen in the consultorio or hospital. In this analysis, one notes that within any given age group the rate of isolation of ETEC from cases of diarrhea is remarkably similar (circa 8%), irrespective of where the case was found.

Isolations of ETEC in the consultorio and hospital were more common in summer months. The distribution of enterotoxin types in ETEC isolated from patients cultured at the consultorio and hospital is the same as encountered in the actively followed cohorts. For example, of the 120 cases of ETEC diarrhea identified among children attending the consultorio, 54 cases (45%) had LT-only, 50 (42%) had ST-only and 16 (13%) had LT/ST infections. The distribution of CFAs was quite similar to what was

found among strains isolated from children with diarrhea in the age cross-sectional cohort. Sixty-seven % of the LT/ST isolates and 82% of the ST-only isolates expressed known CFAs, compared with only 7% of the LT-only isolates. Of all the isolates from the hospital and consultorio with known CFAs, 50% were CFA/I, 45% were CFA/II family and 5% were of the CFA/IV family.

EPEC, EIEC, EHEC and DAEC

The rate isolation of EPEC, EIEC, EHEC and DAEC in children with diarrheal illness attended in the consultorio and the hospital are summarized in Table 6 where they are compared with children followed in the age cross-sectional cohort. Only among children less than 12 months of age are the numbers adequate to compare all three sampling sites. The data suggest that EPEC infections constitute a higher proportion of hospitalized cases of diarrhea than diarrhea seen in the consultorio or detected by household visits. With the other categories, there were either too few cases for comparison or there were no convincing trends.

DISCUSSION

Non-radioactive DNA probe methodology was successfully transferred to a local university microbiology laboratory in Chile. As a consequence, this laboratory in a developing country was able to process the large number of specimens necessary to allow a comprehensive study of the epidemiology of infections due to the different categories of diarrheagenic E. coli. Application of the identical diagnostic methods to E. coli isolates derived from two relatively large and distinct, actively-followed, cohorts of children (one age cross-sectional and the other a birth cohort), as well as to isolates systematically obtained from patients attending the consultorio and the children's hospital that serve Santa Julia, provide a broad perspective to the study. Another relevant feature of the study is that all the recognized categories of diarrheagenic *E. coli* were sought.

We observed that a notable proportion of the episodes of diarrhea in Santa Julia children during summer are due to ETEC and the difference in rate of isolation between cases and controls was highly significant. These data corroborate seroepidemiologic studies reported several years ago (59) which showed that among Santiago children 3-5 years of age the prevalence and mean titer of LT antitoxin (a

measure of past infection with LT-producing ETEC) are high and are similar to those found in Bangladeshi children of the same age. It should be pointed out that several previous studies of diarrheal disease in Latin America (28,29) failed to show a significant difference in the rate of isolation of ETEC in cases as compared with controls.

Considerable microbiologic, clinical and epidemiologic evidence suggests that LT/ST strains are more pathogenic compared with ETEC strains of other toxin types. A large proportion of LT/ST strains express recognized fimbrial colonization factors and fall within a limited array of O:H serotypes (7). Indeed, molecular epidemiologic investigations demonstrate that many LT/ST strains constitute worldwide clones (62,63,63a). Consistent with these observations, most of the LT/ST isolates from Santa Julia were found to express known fimbrial colonization factors. Fimbrial colonization factors generally occur in a much smaller proportion of ST-only strains (64,65), as was seen in this study.

LT-only strains are quite heterogeneous with some being considered pathogenic and others not. Certain LT-only isolates are clearly derived from LT/ST strains following loss of a virulence plasmid that encodes ST and a CFA (68). Repetitive passage of LT/ST strains in the laboratory leads to loss of CFA plasmids from some strains (64). ETEC strain H10407P (O78:H11, LT-only) was derived from ETEC strain H10407 (O78:H11, LT/ST, CFA/I) following spontaneous loss of a plasmid that encodes ST and CFA/I (68). Feeding studies in volunteers showed that the LT/ST parent strain (H10407) caused diarrhea while the LT-only derivative (H10407P) did not (69). LT-only strains of this nature can often be deduced by their O:H serotype.

Certain LT-only strains are inherently pathogenic. One such LT-only strain, E2528-C1 (O25:NM), that was epidemiologically incriminated as the etiologic agent responsible for an outbreak of diarrhea on a cruise ship (70), caused definite (albeit mild) diarrheal illness when fed to volunteers (71). It was subsequently found that this strain expresses CS6. A new fimbrial colonization factor, CS17, has have been identified in LT-only strains of serotypes O114:H21 (72). Finally, it is believed that the majority of remaining LT-only strains, which fit neither of the above descriptions, are either non-pathogenic or of marginal pathogenicity.

Previous studies of ETEC diarrhea in Latin America have usually found LT-only strains to be the most common toxin type and have reported that the rate of isolation between cases and controls is not significantly different (61,72). In Santa Julia, LT-only strains comprised the most common enterotoxin profile. With the large samples involved in the Santa Julia study, the difference in rate of isolation of LT-only ETEC between cases and controls was statistically significant ($p = 0.05$, in the newborn cohort) or near significant ($p = 0.07$, in the age cross-sectional cohort). As a point of comparison, the difference in rate of isolation of LT/ST strains and ST-only strains between cases and controls was highly significant (Tables 3 & 5), generally supporting the concept of greater pathogenicity of these strains. In this context, one might have expected to find a higher proportion of LT/ST and ST-only isolates among cases seen in the consultorio and hospital, since these originate from more severely ill cases; however, this was not the case (Table 7).

Results of challenge studies in volunteers (74,75) and experiments in animal models (76) with carefully characterized strains of EPEC, in conjunction with the recent identification of novel virulence properties (19,20,22), provide incontrovertible evidence that EPEC cause diarrheal illness. Nevertheless, one should recall that in the early 1970s fierce controversy raged over the question of whether or not EPEC were in fact diarrheal pathogens (23,75-79). In that period, the argument largely stemmed from conflicting interpretations of reports of studies where differential rates of isolation of EPEC from cases and controls were compared (79). In their review of the epidemiology of EPEC diarrhea, Levine and Edelman (23) reported that case/control studies generally only show a significant difference in isolation rates of EPEC when children less than 12 months of age are examined. The prospective surveillance of two pediatric cohorts in Santa Julia provides compelling data to support this thesis and confirm the importance of age in relation to the pathogenicity of EPEC. In the newborn cohort the difference in rate of isolation of EPEC between cases and controls was highly significant in infants below 12 months of age but not in older children (Table 5). Even in the age cross-sectional cohort, where the age structure provided only limited data on infants less than six months of age, there was a marked difference in rate of isolation of EPEC between cases and controls among infants < 12

months of age (Table 4).

EIEC infections were not common, being associated with only 2.9% of episodes in the age cross-sectional cohort and with only 1.7% in the newborn cohort. Such rates of isolation are similar to what Taylor et al (78) reported among infants and young children with diarrhea who visited a health care facility in Bangkok. In the two cohorts in Santa Julia the rate of isolation of EIEC was always higher in cases than in controls but the differences overall did not reach significance; only in children $\geq$ 24 months of age in the cross-sectional cohort did the difference approach statistical significance. In this same cohort, Shigella infections, which resemble EIEC in pathogenesis, were three-fold more common than EIEC infections (48).

The hemolytic-uremic syndrome is a well-recognized pediatric entity in Chile (80,81), although the disease is not hyper-endemic as it is in neighboring Argentina (82). Little is known about either the bovine or the human reservoir of EHEC in Chile or about the frequency of occurrence of EHEC diarrheal infections (81). The study in Santa Julia demonstrates that intestinal infection with EHEC occurs but is quite uncommon; EHEC were the least frequently encountered category of diarrheagenic E. coli. The rate of isolation was similar in cases and controls.

Preliminary analysis of the EHEC isolated from diarrhea cases reveals that they carry phage-encoded genes for the expression of Shiga-like toxin 1 or 2 (or both). Thus, the Santa Julia EHEC strains appear capable of causing clinically more severe forms of illness such as hemorrhagic colitis or hemolytic-uremic syndrome. Increasing evidence suggests that genetic host factors determine whether EHEC-infected individuals will manifest clinically severe forms of illness such as hemorrhagic colitis and hemolytic-uremic syndrome. Under any circumstances, the study demonstrates that human EHEC infections occur in Santa Julia, albeit at low incidence, and these infected individuals may comprise a human reservoir for further transmission.

Currently, there is much debate over whether DAEC (defined either phenotypically by diffuse adherence to HEp-2 cells or genotypically by harboring DAEC fimbriae genes) cause diarrhea. In one field study DAEC strains were isolated significantly more often from cases than from controls (12) In

other studies (8,9), including one from Chile (13), they have been found with equal frequency in both cases and controls. A DNA probe was developed to identify DAEC based on detecting the subunit gene for the fimbria that mediates diffuse adherence (11); Levine et al (13) showed the probe to be moderately sensitive and highly specific. Volunteers fed the prototype DAEC strain from which the DNA probe was developed failed to manifest diarrhea, despite ingesting up to 10^{10} viable bacteria with buffer (37).

The data from Santa Julia shed some light on DAEC as enteropathogens. Overall, the difference in rate of isolation between cases and controls in the age cross-sectional cohort was significant ($p = 0.0024$) but the overall relative risk was only 1.4. However, as seen in Table 4, the relative risk appeared to steadily increase with age to reach a value of 2.1 in children $\geq$ 48 months of age. Thus, DAEC may be more important as a diarrheal pathogen in older children. It is of interest that DAEC infections showed the identical seasonality pattern as ETEC infections, a well-accepted enteropathogen. The data from the age cross-sectional cohort in Santa Julia, generated using a DNA probe rather than with the HEp-2 assay, constitute the strongest epidemiologic evidence so far to indicate that DAEC may indeed be pathogenic. There was no significant difference in isolation rate of DAEC between cases and controls in the newborn cohort. However, a difference would not be expected in this cohort if DAEC are primarily pathogenic for older children. Our interpretation of the existing data leads us to conclude that the DAEC fimbria gene probe broadly identifies a category of E. coli that includes some strains capable of causing diarrhea. However, we surmise that there must exist additional virulence properties, such as enterotoxins, present in the strains capable of causing diarrheal illness. Elucidation of these hypothetical additional virulence properties and diagnostic tests to detect their presence would allow more specific identification of DAEC pathogens.

Enteroaggregative E. coli (EAggEC), the newest category of diarrheagenic E. coli (8-10,30,31) has been incriminated as an important cause of persistent diarrhea, a syndrome which carries a poor prognosis when encountered in children in less-developed countries. While the first report associating EAggEC and pediatric diarrhea was carried out in Santiago, Chile (13), in the current study no

significant association was found, either for diarrhea overall or for persistent diarrhea. In the original report from Chile in which EAggEC were incriminated, the ill children were seen in hospital and in a health center and the identification of EAggEC was made by HEp-2 cell assay. In this study EAggEC were sought only in children followed with active surveillance via household visits (which generally detects milder illnesses than clinic-based passive surveillance) and EAggEC were identified using a DNA probe that detects the EAggEC virulence plasmid. It is also possible that the failure to encounter EAggEC significantly in association with diarrhea is due to year to year secular variations.

In analyzing the incidence rates of diarrheal illness due to the different categories of diarrheagenic E. coli in Santa Julia and in comparing these data to reports from other geographic areas, one must keep in mind the particular characteristics of this field site in Santiago. Santa Julia is representative of peri-urban communities undergoing rapid development. While housing is ramshackle and households are severely crowded, virtually all households in Santa Julia have access to bacteriologically-monitored water (68% have water taps inside) and 64% of households have a toilet inside the home. Thus, the high incidence of ETEC and EPEC diarrhea observed in Santa Julia demonstrate that considerable transmission of these pathogens is occurring despite the availability of potable water. This points to the likelihood of contaminated food serving as vehicles of transmission to children. The fact that more than half the households in Santa Julia lack a refrigerator to preserve food when ambient temperatures are high and that the seasonal peak of ETEC, EPEC and DAEC diarrhea occurs in summer are compatible with food-borne transmission.

As one might anticipate in a poor community to which bacteriologically-controlled water has been provided, the overall incidence of pediatric diarrhea in children in Santa Julia (48) is low compared with reports from less-developed regions of South America (40,41). Nevertheless, a high incidence of shigellosis persists in Santa Julia along with the relatively high incidence of diarrheal illness due to certain categories of diarrheagenic E. coli as quantitated in this report. This emphasizes that additional interventions and infrastructure will have to be instituted in order to diminish further the transmission of bacterial enteropathogens. A particularly important advance may be the acquisition of a

means tc refrigerate food. Vaccines against certain categories of diarrheagenic *E. coli*, such as ETEC, might constitute a relevant intervention for the pediatric population of Santa Julia. Moreover, Santa Julia is an attractive site to undertake field trials to assess the efficacy of such vaccines.

REFERENCES

1. Snyder JD, Merson MH. The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull WHO 1982;60:605-613

2. Levine MM, Losonsky G, Herrington D, Kaper JB, Tacket CO, Rennels MB, Morris JG. Pediatric diarrhea: the challenge of prevention. Pediat Infect Dis J 1986;5 supplement:S29-S43.

3. Diarrhoeal Diseases Control Programme, World Health Organization. Biomedical and Epidemiological Research Priorities of Global Scentific Working Groups. WHO/CDD/86.8 Rev.1 (1987). Geneva, 1987.

4. Levine MM. Vaccines against enteric infections. Lancet 335: 958-961, 1990.

5. Bray J. Isolation of antigenically homogeneous strains of *Bact coli neopolitanum* in summer diarrhea of infants. J Pathol Bacteriol 1945;57:239-247.

6. Giles C, Sangster MB. An outbreak of infantile gastroenteritis in Aberdeen. J Hyg 1948;46:1-9.

7. Levine MM. Escherichi coli that cause diarrhea: Enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J Infect Dis 1987;155:377-389.

8. Bhan MK, Raj P, Levine MM, Kaper JB, Bhandari N, Srivastava R, Kumar R, Sazawal S. Enteroaggregative Escherichia coli associated with persistent diarrhea in a cohort of rural children in India. J Infect Dis 159: 1061-1064, 1989.

9. Cravioto A, Tello A, Navarro A, Ruiz J, Villafan H, Uribe F, Eslava C. Association of Escherichia coli HEp-2 adherence patterns with type and duration of diarrhoea. Lancet 1991;337:262-264.

10. Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial PA, Levine MM. Patterns of adherence of diarrheagenic Escherichia coli to HEp-2 cells. Pediat Infect Dis J 1987;6:829-831.

11. Bilge SS, Clausen CR, Lau W, Moseley SL. Molecular characterization of a fimbr[illegible] adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to [illegible]2 cells. J Bacteriol 1989;171:4281-4289.

12. Giron J, Jones T, Millan-Velasco F, Muñoz-castro E, Fry J, Frankel G, Moseley S, [illegible]dry [illegible], Kaper JB, Schoolnik GK, Riley LW. Diffuse-adhering *Escherichia coli* (DAEC) as a [illegible]tive

cause of diarrhea in Mayan children in mexico. J Infect Dis 1991;164:693-703.

13. Levine MM, Prado V, Robins-Browne RM, Lior H, Kaper JB, Moseley S, Gicquelais K, Nataro JP, Vial P, Tall B. DNA probes and HEp-2 cell adherence assay to detect diarrheagenic Escherichia coli. J Infect Dis 1983;158:224-228.

14. Hale TL, Oaks EV, Formal SB. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of Shigella spp. and enteroinvasive Escherichia coli. Infect Immun 1985;50:620-629.

15. Hale TL, Sansonetti PJ, Schad PA, Austin S, Formal SB. Characterization of virulence plasmids and virulence-associated, plasmid-coded outer membrane proteins in Shigella flexneri, Shigella sonnei and Escherichia coli. Infect Immun 1983;40:340-350.

16. Fasano A, Kay BA, Russell RG, Maneval DR, Levine MM. Enterotoxin and cytotoxin production by enteroinvasive Escherichia coli. Infect Immun 1990;58:3717-3723.

17. DuPont HL, Formal SB, Hornick RB, Snyder MJ, Libonati JP, Sheahan DG, LaBrec EH, Kalas JP. Pathogenesis of Escherichia coli diarrhea. N Engl J Med 1971;285:1-9.

18. Marier R, Wells JG, Swanson RC, Callahan W, Mehlman IJ. An outbreak of enteropathogenic Escherichia coli foodborne disease traced to imported french cheese. Lancet 1973;II:1376-1378.

19. Jerse AE, Yu J, Tall BD, Kaper JB. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells Proc Nat Acad Sci USA 1990;87:7839-7843.

20. Nataro JP, Scaletsky ICA, Kaper JB, Levine MM, Trabulsi LR. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. Infect Immun 1985;48:378-383.

21. Jerse AE, Kaper JB. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect Immun 1991;59:4302-4309.

22. Giron JA, Ho ASY, Schoolnik GK. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 1991;254:710-713.

23. Levine MM, Edelman R. Enteropathogenic *Escherichia coli* of classical serotypes associated with infant diarrhea- epidemiology and pathogenesis. Epidemiol Rev 1984;6:31-51.

24. Karch H, Heeseman J, Laufs R, O'Brien AD, Tacket CO, Levine MM. A plasmid of enterohemorrhagic Escherichia coli 0157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect Immun 1987;55:455-461.

25. O'Brien AD, Marques LRM, Kerry CF, Newland JM, Holmes RK. Shiga-like toxin converting phage of enterohemorrhagic Escherichia coli strain 933. Microbial Path 1989;6:381-390.

26. Tzipori S, Karch H, Wachsmuth KI, Robins-Browne RM, O'Brien AD, Lior H, Cohen ML, Smithers J, Levine MM. Role of a 60-megadalton plasmid and Shiga-Like toxins in the pathogenesis of infection caused by enterohemorrhagic Escherichia coli 0157:H7 in gnotobiotic piglets. Infect Immun 1987;55:3117-3125.

27. Yu J, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli*. Molec Microbiol 1992;6:411-417.

28. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol Rev 1991;13:60-98.

29. Pavia AT, Nichols CR, Green DP, Tauxe RV, Mottice S, Greene KD, Wells JG, Siegler RL, Brewer ED, Hannon D, Plake PA. Hemolytic-uremiv syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally-retarded persons: clinical and epidemiologic observations. J Pediat 1990;116:544-651.

30. Vial P, Robins-Browne RM, Lior P, Prado V, Nataro JP, Elsayed A, Levine MM. Characterization of enteroadherent-aggregative *Escherichia coli* (EA-AggEC): a putative agent of diarrheal disease. J Infect Dis 1988;158:70-79.

31. Vial PA, Mathewson JJ, DuPont HL, Guers L, Levine MM. Comparison of two assay methods

for patterns of adherence to HEp-2 cells of Escherichia coli from patients with diarrhea. J Clin Microbiol 1990;28:882-885.

32. Yamamoto T, Endo S, Yokota T, Echeverria P. Characterstics of adherence of enteroaggregative *Escherichia coli* to human and animal mucosa. Infect Immun 1991;59:3722-3739.

33. Yamamoto T, Echeverria P, Yokota T. Drug resistance and adherence to human intestines of enteroaggregative *Escherichia coli*. J Infect Dis 1992;165:744-749.

34. Nataro I&I

35. Savarino SJ, Fasano A, Robertson DC, Levine MM. Enteroaggregative Escherichia coli elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. J Clin Invest 1991;87:1450-1455.

36. Wanke C, Schorling JB, Barrett LJ, DeSouza MA, Guerrant RL. Potential role of adherence traits of *Escherichia coli* in persistant diarrhea in an urban Brazilian slum. Pediat Infect Dis J 1991;10:746-751.

37. Tacket CO, Moseley SL, Kay BA, Losonsky GA, Levine MM. Challenge studies in volunteers using Escherichia coli strains with diffuse adherence to HEP-2 cells. J Infect Dis 1990;162:550-552.

38. Gomes, TA, Blake PA, Trabulsi LR. Prevalence of Escherichia coli strains with localized, diffuse, and aggregative adherence to HeLa cells in infants with diarrhea and matched controls. J Clin Microbiol 1989;27:266-269.

39. Black RE, Brown KH, Becker S, Abdul Alim ARM, Huq I. Longitudinal studies of infectious diseases and physical growth in rural Bangladesh. II. Incidence of diarrhea and association with known pathogens. Am J Epidemiol 1982;115:315-324.

40. Black RE, Lopez de Romana, Brown KH, et al. Incidence and etiology of infantile diarrhea and major routes of transmission in Huascar, Peru. Am J Epidemiol 1989;129:785-99.

41. Guerrant RL, Kirchhoff LV, Shields DS, Nations MK, Leslie J, de Sousa MA, Araujo JG, Correia

LL, Sauer KT, McClelland KE, Trowbridge FL, Hughes JM. Prospective study of diarrheal illnesses in Northeastern Brazil: patterns of disease, nutritional impact, etiologies, and risk factors. J Infect Dis 1983; 148:986-997.

42. Cravioto, A., Reyes, R.E., Ortega, R., Fernandez, G., Hernandez, R., Lopez, D. 1988. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. Epidemiol. Infect. 101:123-134.

43. Bhan MK, Bhandari N, Sazawal S, Clemens J, Raj P, Levine MM, Kaper JB. Descriptive epidemiology of persistent diarrhoea among young children in rural northern India. Bull Wld Hlth Org 1989;67:281-288.

44. Araya M, Figueroa G, Espinoza J, Montesinos N, Spencer E, Brunser O. Acute diarrhgoeal disease in children under 7 years of age in a peri-urban slum of Santiago, Chile. J Hyg 1985;95:457-467.

45. Rowland SGJG, LLoyd-Evans N, Williams K, Rowland MGM. The etiology of diarrhoea studied in the community in young urban Gambian children. J Diar Dis Res 1985;3:7-13.

46. Gicquelais KG, Baldini MM, Martinez J, Maggi L, Martin WC, Prado V, Kaper JB, Levine MM. Practical and economical method for using biotinylated DNA probes with bacterial colony blots to identify diarrhea-causing Escherichia coli. J Clin Micro 1990;28: 2485-2490.

47. Oprandy JJ, Thornton SA, gardiner CH, Burr D, Batchelor R, Bouregois L. Alkaline phosphatase-conjugated oligonucleotide probes for enterotoxigenic *Escherichia coli* in travelers to South America and West Africa. J Clin Microbiol 1988;26:92-95.

48. Ferreccio C, Prado V, Ojeda A, Cayazzo M, Abrego P, Guers L, Levine MM. Epidemiologic patterns of acute diarrhea and endemic Shigella infections in a poor periurban setting in Santiago, Chile. Am J Epidemiol 1991;134:614-627.

49. DiJohn D, Levine MM. Treatment of diarrhea. Infect Dis Clin N Amer 1988;2:719-45.

50. Lanata CF, Kaper JB, Baldini MM, Black RE, Levine MM. Sensitivity and specificity of DNA probes with the stool blot technique for detection of Escherichia coli enterotoxins. J Infec Dis

1985;152:1087-1090.

51. Wood PK, Morris JG jr, Small PLC, Sethabutr O, Toledo MRE, Trabulsi L, Kaper JB. Comparision of DNA probes with the Sereny test for identification of invasive Shigella and Escherichia coli strains. J Clin Microbiol 1986; 24:498-500.

52. Levine MM, Xu J, Kaper JB, Lior H, Prado V, Tall B, Nataro J, Karch H, Wachsmuth K. A DNA probe to identify enterohemorrhagic Escherichia coliof O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J Infect Dis 1987; 156:175-182

53. Baudry B, Savarino SJ, Vial PA, Kaper JB, Levine MM. A sensitive and specific DNA probe to identify enteroaggregative Escherichia coli, a recently-discovered diarrheal pathogen. J Infect Dis 1990;161:1249-1251.

54. Nataro JP, Baldini MM, Kaper JB, Black RE, Bravo N, Levine MM. Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J Infect Dis 1985; 152:560-5

55. Newland JW, Neill RJ. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J Clin Microbiol 1988;26:1292-1297.

56. Faundez G, Figueroa G, Troncoso M, Cabello FC. Characterization of enteroinvasive Escherichia coli strains isolated from children with diarrhea in Chile. J Clin Microbiol 1988;26:928-932.

57. Maneval colony blot immunoassay

58. Ewing WH. Edwards and Ewing's identification of enterobacteriaceae, 4th ed.. Elsevier Science Publishing, Inc. New York.

58a. Taylor DN, Echeverria P, Sethabutr O, Pitarangsi C, Leksomboon U, Blacklow NR, Rowe B, Gross R, Cross J. Clinical and microbiologic features of *Shigella* and enteroinvasive *Escherichia coli* infections detected by DNA hybridization. J Clin Microbiol 1988;26:1362-1366.

59. Levine MM, Young CR, Black RE, Takeda Y, Finkelstein RA. Enzyme-linked immunosorbent assay to measure antibodies to purified heat-labile enterotoxins from human and porcine strains of Escherichia coli and to cholera toxin: application in serodiagnosis and seroepidemiology. J

Clin Microbiol 1985; 21:174-179.

60. Spencer HC, Wells JG, Gary WG, Sondy J, Puhr ND, Feldman RA. Diarrhea in a non-hospitalized rural Salvadoran population: the role of enterotoxigenic Escherichia coli and rotavirus. Am J Trop Med Hyg 1980; 29:246-253.

61. Pickering LK, Evans DJ Jr, Muñoz O, DuPont HL, Coell-Ramirez P, Vollet JJ, Conklin RH, Olarte J. Prospective study of enteropathogens in children with diarrhea in Houston and Mexico. J Pediat 1978;93:383-388.

62. Orskov F, Orskov I. Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the *Enterobacteriaceae* and other bacteria. J Infect Dis 1983;148:346-357.

63. Willshaw GA, Smith HR, McConnell MM, Barclay EA, Krnjulac J, Rowe B. Genetic and molecular studies of plasmids coding for colonization factor antigen I and heat-stable enterotoxin in several *E. coli* serotypes. Infect Immun 1982;37:858-868.

63a Danbara H, Komase K, Arita H, Abe H, Yoshikawa M. Molecular analysis of enterotoxin plasmids of enterotoxigenic Escherichia coli of 14 different O serotypes. Infect Immun 1988;56:1513-1517.

64. Levine MM, Ristaino P, Sack RB, Kaper JB, Orskov F, Orskov I. Colonization factor antigens I and II and type 1 somatic pili in enterotoxigenic Escherichia coli: relation to enterotoxin type. Infect Immun 1983;39:889-897.

65. Gothefors L, Ahren C, Stoll B, Barua D, Orskov F, Salek MA, Svennerholm A-M. Presence of colonization factor antigens on fresh isolates of fecal *Escherichia coli*. J Infect Dis 1987;155:377-389.

66. Binsztein N, Jouve MJ, Viboud GI, Lopez Moral L, Rivas M, Orskov I, Ahren C, Svennerholm A-M. Colonization factors of enterotoxigenic *Escherichia coli* isolated from children with diarrhea in Argentina. J Clin Microbiol 1991;29:1893-1898.

67. Thomas LV, Rowe B. The occurrence of colonization factors (CFA/I, CFA/II and E8775) in

enterotoxigenic *Escherichia coli* from various countries in South East Asia. Med Microbiol Immunol 1982;171:85-90.

68. Evans DG, Silver RP, Evans DJ Jr, Chase DG, Gorbacj SL. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect Immun 1975;12:656-667.

69. Satterwhite TK, Evans DG, DuPont HL, Evans DJ Jr. Role of Escherichia coli colonisation factor antigen in acute diarrhea. Lancet II:1978;181-184.

70. Lumish RM, Ryder RW, Anderson DC, Wells JG, Puhr ND. Heat-labile enterotoxigenic *Escherichia coli* induced diarrhea aboard a Miami-based cruise ship. Am J Epidemiol 1980;111:432-436.

71. Levine MM, Nalin DR, Hoover DL, Bergquist EJ, Hornick RB, Young CR. Immunity to Enterotoxigenic Escherichia coli. Infect Immun 1979;23:729-736.

72. McConnell MM, Hibbard M, Field AM, Chart H, Rowe B. Characterization of a new colonization factor (CS17) from a human enterotoxigenic *Escherichia coli* of serotype O114:H21 which produces only heat-labile enterotoxin. J Infect Dis 1990;161:343-347.

73. Reis MHL, Guth BEC, Gomes TAT, Murahivschi J, Trabulsi L. Frequency of *Escherichia coli* strains producing heat-labile toxin or heat-stable toxin or both in children with and without diarrhea in Sao Paulo. Infect Immun 1982;15:1062-1064.

74. Levine MM, Bergquist EJ, Nalin DR, Waterman DH, Hornick RB, Young CR, Sotman S, Rowe B. Escherichia coli strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet 1978;I:III9-II22.

75. Levine MM, Nataro JP, Baldini MM, Kaper JB, Black RE, Clements ML, O'Brien AD. The diarrheal response of humans to some classical serotype enteropathogenic Escherichia coli is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis 1985;152:550-559.

76. Tzipori S, Robins-Browne RM, Gonis G, Hayes J, Withers M, McCartney E. Enteropathogenic

Escherichia coli enteritis: evaluation of the gnotobiotic piglet as a model of human infection. Gut 1985;26:570-578.

77. Sack RB. Serotyping of *E. coli*. Lancet 1976;I:1132.

78. French GL. Enteropathogenic *E. coli*. Lancet 1976;I:1411-1412.

79. Gangarosa EJ, Merson MH. Epidemiologic assessment of the relevance of the so-called enteropathogenic serogroups of *Escherichia coli* in diarrhea.

80. Cordero J, Baeza J, Fielbaum O, et al. Sindrome hemolitico uremico. Rev Chil Pediatr 1984; 55:29-33.

81. Cordovéz A, Prado V, Maggi L, Cordero J, Martinez J, Misraji A, Rios R, Soza G, Ojeda A, Levine MM. Enterohemorrhagic Escherichia coli associated with the hemolytic-uremic syndrome in Chilean children. J Clin Microbiol 1992; in press.

82. Giantonio C, Vitacco M, Mendilaharzu F, Rutty A, Mendilharzu J. The hemolytic-uremic syndrome. J Pediatr 1964; 64:478-491.

TABLE 1. Isolation of diarrheagenic *E. coli* in cases of diarrhea and controls in an age cross-sectional cohort of children 0 - 59 months of age followed prospectively by active surveillance involving twice weekly household visits: Santa Julia, Santiago, Chile, December 1, 1986 to May 31, 1989.

Trimester	Cases	Controls	ETEC+		EPEC+		EIEC+		EHEC+		DAEC+		EAggEC	
			Cases	Ctrls	Cases	Ctrls	Cases	Ctrls	Cases	Ctrls	Cases	Ctrls	Cases	Ctrls
Dec. 1986-Feb. 1987	145 (143)*	145 (141)	25	11	4	2	2	6	1	1	31	15	50	35
March - May	154 (145)	145 (144)	21	15	10	5	6	2	1	3	32	27	18	30
June - Aug.	78 (70)	71 (66)	3	2	1	3	3	2	1	1	22	18	2	8
Sept. - Nov.	94 (79)	82 (78)	3	2	0	0	1	1	5	1	13	6	17	19
Dec. 1987-Feb. 1988	142 (132)	134 (134)	21	13	5	4	5	2	4	1	26	17	25	38
March - May	134 (125)	127(124)	14	6	4	5	6	3	4	2	24	21	12	8
June - Aug.	75 (68)	69 (67)	5	4	1	3	1	1	0	3	5	1	10	11
Sept. - Nov.	111 (106)	108 (104)	18	4	3	0	0	0	0	4	7	2	16	13
Dec. 1988 - Feb. 1989	145 (140)	140 (136)	19	10	14	11	3	3	0	1	11	11	17	22
March - May	100 (73)	36 (69)	4	7	5	4	4	0	0	2	8	7	8	12
Total	1178(1081)	1090(1063)	133[a]	74[b]	47[c]	37[d]	31[e]	20[f]	16[g]	20[h]	179[i]	126[j]	175[k]	196[l]

* (Number of episodes where cultures were tested with DNA probes)

+ ETEC = enterotoxigenic E. coli; EPEC = enteropathogenic E. coli; EIEC = enteroinvasive E. coli; EHEC enterohemorrhagic E. coli; DAEC = E. coli that manifest the diffuse pattern of adherence to HEp-2 cells.

p values by Chi square:

a vs b, p = 0.000039 — e vs f, p = NS — i vs j, p = 0.0024

c vs d, p = NS — g vs h, p = NS — k vs l, p = NS

TABLE 2. Number of episodes and incidence of diarrhea due to the different categories of diarrheagenic *Escherichia coli* in two cohorts of children under active household surveillance: Santa Julia, Santiago, Chile

Age group (mos.)	Child mos. of observation	All diarrhea: No.	All diarrhea: Incidence per 12 child mos.	ETEC* No.	ETEC* Incidence per 12 child mos.	EPEC* No.	EPEC* Incidence per 12 child mos.	EIEC* No.	EIEC* Incidence per 12 child mos.	EHEC* No.	EHEC* Incidence per 12 child mos.	DAEC* No.	DAEC* Incidence per 12 child mos.	EAggEC* No.	EAggEC* Incidence per 12 child mos.
Age cross-sectional cohort†:															
0-11	937	173	2.1	22	0.28	20	0.26	3	0.04	4	0.05	20	0.26	43	0.55
12-23	1745	303	1.9	34	0.23	12	0.08	8	0.06	5	0.03	51	0.35	51	0.35
24-35	2287	267	1.3	35	0.18	7	0.04	6	0.03	2	0.01	44	0.23	37	0.19
36-47	2492	255	1.1	26	0.13	5	0.02	8	0.04	3	0.01	33	0.16	27	0.13
≥ 48	2573	160	0.8	14	0.08	3	0.01	6	0.03	2	0.01	31	0.15	19	0.09
Newborn cohort††:															
0-11	1523	320	2.5	19	0.15	21	0.17	3	0.02	4	0.03	49	0.39	61	0.48
12-23	1412	354	3.0	33	0.27	10	0.08	10	0.08	6	0.05	38	0.32	35	0.30

* ETEC = enterotoxigenic *E. coli*; EPEC = enteropathogenic *E. coli*; EIEC = enteroinvasive *E. coli*; EHEC enterohemorrhagic *E. coli*; DAEC = *E. coli* that exhibit diffuse adherence to HEp-2 cells; EAggEC = enteroaggregative *E. coli*.

† Approximately 360 children followed from December 1, 1986 to May 31, 1989

†† Approximately 144 children (12 enrollees monthly for 12 months) followed from birth through 24 months of age, March 1987 through February 1990

TABLE 3. Isolation of enterotoxigenic E. coli in cases and controls, by age group, in an age cross-sectional cohort of children under active household surveillance: Santa Julia, Santiao, Chile, December 1, 1986 to May 31, 1989

Category of E. coli	Age Group (mos.)	Cases		Controls		p value	RR*
All ETEC+							
	0 - 11	22/162	(13.5%)	10/159	(6.3%)		2.1
	12 - 23	34/281	(12.1%)	24/275	(8.7%)		1.4
	24 - 35	35/251	(13.9%)	13/249	(5.2%)		2.7
	36 - 47	28/230	(12.2%)	17/223	(7.6%)		1.6
	≥ 48	14/157	(8.9%)	10/157	(6.4%)		1.4
	TOTAL	133/1081	(12.3%)	74/1063	(7.0%)	0.000039	1.8
LT/ST strains	0 - 11	3/162	(1.9%)	1/159	(0.6%)		1.2
	12 - 23	8/281	(2.8%)	5/275	(1.8%)		1.6
	24 - 35	9/251	(3.9%)	2/249	(0.8%)		4.9
	36 - 47	7/230	(3.0%)	2/223	(0.9%)		3.3
	≥ 48	4/157	(2.5%)	2/157	(1.3%)		1.9
	TOTAL	31/1081	(2.9%)	9/1063	(0.8%)	0.00097	3.6
ST only strains	0 - 11	7/162	(4.3%)	2/159	(1.3%)		3.3
	12 - 23	8/281	(2.8%)	5/275	(1.8%)		1.6
	24 - 35	10/251	(4.0%)	2/249	(0.8%)		5.0
	36 - 47	5/230	(2.2%)	4/223	(1.8%)		1.2
	≥ 48	4/157	(2.5%)	2/157	(1.3%)		1.9
	TOTAL	34/1081	(3.1%)	15/1063	(1.4%)	0.011	2.2
LT only strains	0 - 11	12/162	(7.4%)	6/159	(3.8%)		1.9
	12 - 23	18/281	(6.4%)	17/275	(6.2%)		1.1
	24 - 35	16/251	(6.4%)	8/249	(3.2%)		2.0
	36 - 47	14/230	(6.1%)	9/223	(4.0%)		1.5
	≥ - 48	6/157	(3.8%)	6/157	(3.8%)		1.0
	TOTAL	66/1081	(6.1%)	46/1063	(4.3%)	0.080	1.4

* RR = relative risk

\+ In two of the 133 episodes of ETEC diarrhea both LT-only and ST-only strains were isolated. Similarly, from four of the 74 controls whose cultures yielded ETEC, both LT-only and ST-only strains were isolated. These isolations are included in the totals but are excluded from the sub-analyses by toxin profile shown in this table.

TABLE 4. Isolation of diarrheagenic E. coli in cases and controls, by age group, in an age cross-sectional cohort of children under active household surveillance: Santa Julia, Santiago, December 1, 1988 to May 31, 1989

Category of E. coli	Age Group (mos.)	Cases		Controls		RR*
ETEC+						
	0 - 11	22/162++	(13.6%)	10/159	(6.3%)	2.2
	12 - 23	34/281	(12.1%)	24/275	(8.7%)	1.4
	24 - 35	35/251	(13.9%)	13/249	(5.2%)	2.7
	36 - 47	28/230	(12.2%)	17/223	(7.6%)	1.6
	≥ 48	14/157	(8.9%)	10/157	(6.4%)	1.4
	TOTAL	133/1081	(12.3%)	74/1063	(7.0%)	1.8
EPEC						
	0 - 11	20/162	(12.3%)	10/159	(6.3%)	2.0
	12 - 23	12/281	(4.3%)	11/275	(4.0%)	1.1
	24 - 35	7/251	(2.8%)	10/249	(4.0%)	0.7
	36 - 47	5/230	(2.2%)	5/223	(2.2%)	1.0
	≥ 48	3/157	(1.9%)	1/157	(0.6%)	3.2
	TOTAL	47/1081	(4.3%)	37/1063	(3.5%)	1.2
EIEC						
	0 - 11	3/162	(1.9%)	4/159	(2.5%)	0.8
	12 - 23	8/281	(2.8%)	7/275	(2.5%)	1.1
	24 - 35	6/251	(2.4%)	4/249	(1.6%)	1.5
	36 - 47	8/230	(3.5%)	3/223	(1.3%)	2.7
	≥ 48	6/157	(3.8%)	2/157	(1.3%)	2.9
	TOTAL	31/1081	(2.9%)	20/1063	(1.9%)	1.5
EHEC						
	0 - 11	4/162	(2.5%)	3/159	(1.9%)	1.3
	12 - 23	5/281	(1.8%)	7/275	(2.5%)	0.7
	24 - 35	2/251	(0.8%)	4/249	(1.6%)	0.5
	36 - 47	3/230	(1.3%)	2/223	(0.9%)	1.4
	≥ 48	2/157	(1.3%)	4/157	(2.5%)	0.5
	TOTAL	16/1081	(1.5%)	20/1063	(1.9%)	0.8
DAEC						
	0 - 11	20/162	(12.3%)	18/159	(11.3%)	1.1
	12 - 23	51/281	(18.1%)	41/275	(14.9%)	1.2
	24 - 35	44/251	(17.5%)	31/249	(12.4%)	1.4
	36 - 47	33/230	(14.3%)	21/223	(9.4%)	1.5
	≥ 48	31/157	(19.7%)	15/157	(9.6%)	2.1
	TOTAL	179/1081	(16.6%)	126/1063	(11.9)	1.4
EAggEC						
	0 - 11	43/162	(26.6%)	34/159	(21.4%)	1.2
	12 - 23	51/281	(18.1%)	68/276	(24.6%)	0.7
	24 - 35	37/251	(14.7%)	46/250	(18.4%)	0.8
	36 - 47	27/230	(11.7%)	32/223	(14.3%)	0.8
	≥ 48	19/156	(12.2%)	19/156	(12.2%)	1.0
	TOTAL	177/1080	(16.4)	199/1064	(18.7%)	0.9

* RR = relative risk

\+ All ETEC infections of any toxin profile (i.e., LT/ST, LT or ST.)

++ No. positive/No. of episodes from which E. coli were tested by DNA probes.

TABLE 5. Isolations of diarrheagenic E. coli from episodes of diarrhea or from matched controls in the newborn cohort followed from birth to 24 months of age: Santa Julia, Santiago, Chile, March, 1987 to February, 1990.

Isolations	Cases Age Group (mos.) 0-2	3-5	6-8	9-11	12-14	15-17	18-20	21-23	Total	Controls Age Group (mos.) 0-2	3-5	6-8	9-11	12-14	15-17	18-20	21-23	Total
Total no. episodes cultured	44	75	79	116	102	107	74	65	662	44	76	79	119	101	105	73	61	658
ETEC																		
LT/ST	0	0	0	0	2	2	0	1	5	0	0	2	0	1	0	0	0	3
ST-only	0	1	1	4	6	5	2	2	21	0	0	0	1	1	2	2	2	6
LT-only	3	4	3	1	3	1	1	4	20	2	2	0	3	1	0	1	1	10
EPEC	3	7	6	5	3	1	3	3	31	2	1	3	2	3	4	3	2	20
EIEC	0	0	1	1	4	4	4	1	12	0	0	0	1	2	2	4	0	9
EHEC	0	1	3	0	2	1	2	1	10	0	1	3	1	5	3	3	1	17
DAEC	11	11	8	19	10	9	13	6	87	7	18	16	19	10	0	10	7	97
EaggEC	7	19	18	17	8	9	8	10	96	8	15	18	15	8	12	5	6	87

TABLE 6. A comparison of the relative frequency of isolation of diarrheagenic *E. coli* by sampling site and by age group: Santa Julia, December 1, 1986 to May 31, 1989

Sampling site	Age Group (months)	No. diarrheal episodes (cultured)*	No. episodes due to ETEC	No. episodes due to EPEC	No. episodes due to EIEC	No. episodes due to EHEC	No. episodes due to DAEC	No. episodes due to EAggEC
Active surveillance (age cross-sectional cohort)								
	0 - 11	173 (155)	15 (9.7%)	13 (8.4%)	2 (1.3%)	2 (1.3%)	10 (6.5%)	
	12 - 23	303 (270)	23 (8.5%)	7 (2.6%)	7 (2.6%)	4 (1.5%)	28 (10.4%)	
	24 - 35	267 (229)	20 (8.7%)	5 (2.2%)	3 (1.3%)	0 -	20 (8.7%)	
	36 - 47	255 (210)	16 (7.6%)	5 (2.4%)	5 (2.4%)	1 (0.5%)	20 (9.5%)	
	≥ 48	180 (147)	11 (7.5%)	1 (0.7%)	2 (1.4%)	2 (1.4%)	21 (14.3%)	
	TOTAL	1178 (1011)	85 (8.4%)	31 (3.1%)	19 (1.9%)	9 (0.9%)	99 (9.8%)	
Passive surveillance:								
Consultorio	0 - 11	606 (552)	34 (6.2%)	39 (7.1%)	4 (0.7%)	3 (0.5%)	51 (9.2%)	
	12 - 23	583 (543)	51 (9.4%)	21 (3.9%)	8 (1.5%)	1 (0.2%)	68 (12.5%)	
	24 - 35	228 (205)	19 (9.3%)	3 (1.5%)	9 (4.4%)	2 (1.0%)	23 (11.2%)	
	36 - 47	126 (99)	9 (9.1%)	8 (8.1%)	4 (4.0%)	1 (1.0%)	10 (10.1%)	
	≥ 48	115 (102)	9 (9.8%)	1 (1.0%)	4 (3.9%)	0 -	15 (14.7%)	
	TOTAL	1658 (1501)	122 (8.1%)	72 (4.8%)	29 (1.9%)	7 (0.5%)	167 (11.1%)	
Hospital	0 - 11	210 (194)	16 (8.2%)	28 (14.4%)	0 -	0 -	27 (13.9%)	
	12 - 23	40 (33)	2 (6.1%)	2 (6.1%)	0 -	1 (3.0%)	1 (3.0%)	
	24 - 35	19 (16)	1 (6.3%)	1 (6.3%)	0 -	0 -	3 (18.8%)	
	36 - 47	4 (4)	1 (25.0%)	0 -	0 -	0 -	0 -	
	≥ 48	2 (1)	0 (0.0%)	0 -	0 -	0 -	0 -	
	TOTAL	275 (248)	20 (8.1%)	31 (12.5%)	0 -	1 (0.4%)	31 (12.5%)	

* Only one culture per child per episode was obtained from children seen at the consultorio and at the hospital. Therefore, for purposes of comparison, only the first culture from the active surveillance cohort was included in the analysis.